MOLECULAR AND PHYSIOLOGICAL STUDIES OF *PISUM* MUTANTS

by

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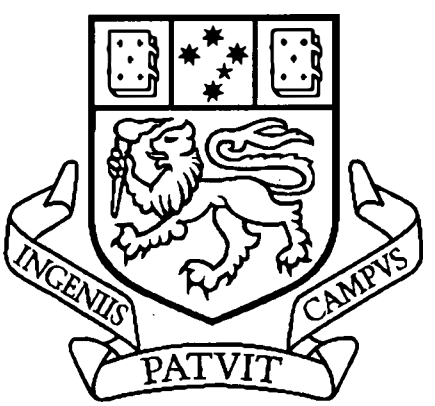

UNIVERSITY OF TASMANIA

Declaration

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Shona L. Batge

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Abstract

The characterisation of developmental mutants generates insights into plant growth processes. This thesis presents a molecular investigation of three phytochrome A mutants of *Pisum sativum* L. (garden pea), and a study of the influence of abscisic acid (ABA) on seed development in the gibberellin (GA)-deficient mutant *lh-2*.

Phytochromes are plant biliproteins which sense red (R, 600 - 700 nm) and far-red (FR, 700 - 760 nm) light and act in concert with other photoreceptors to influence developmental processes, such as germination, seedling de-etiolation, daylength perception and transition to flowering, in response to environmental cues. Two allelic mutants at the *fun1* locus are deficient in phytochrome A. Point mutations have now been identified within the *PHYA* structural gene of both *fun1-1* and *fun1-2*. These cause premature stop codons, predicted to result in production of a truncated and inactive phytochrome A protein in the mutant plants. This is consistent with the demonstration that *fun1-2* plants lack immunodetectable PHYA, and with the previously described phenotypes. Co-segregation between the *fun1-2* mutant phenotype and the identified molecular lesion confirms that the *fun1* mutant phenotype is the direct result of disruption to the *PHYA* structural gene. The *fun1* mutants can now be used confidently in future rigorous analyses of the physiological and developmental roles of the phytochrome family in pea.

The AF05 mutant phenotype is similar to that described for transgenic phytochrome A-overexpressing lines in other species. Results presented in this thesis indicate that there is no mutation in the *PHYA* gene of this mutant, either in the coding region or promoter. Possible explanations for the phenotype are discussed.

In *Arabidopsis* and tomato, phytochrome A mediates a FR-light induced block of greening when seedlings are transferred from FR to white light. This response is considered with regard to the phytochrome A mutants of pea.

Gibberellin (GA)-deficient *lh-2* mutant plants of pea are dwarf in stature and show increased seed abortion and decreased seed weight, compared with seeds of the wild type. This aberrant seed development is associated with reduced levels of active GA, and with an accumulation of abscisic acid (ABA) in young seeds. Hormonal influence on seed development was investigated by construction of an

lh-2 wil double mutant. The *wil* mutation blocks ABA biosynthesis, and reduces ABA levels in young seeds by 10-fold, such that introduction of the *wil* mutation reduces the endogenous ABA levels in young *lh-2* seeds. However, this fails to rescue the seeds from abortion. This indicates that the effects of *lh-2* on seed development are not mediated through increased ABA levels, and is consistent with previous evidence that GAs are the controlling factor underlying the *lh-2* seed phenotype in pea.

Contents

Abbreviations

ABA	abscisic acid
B	blue light
bp	base pairs
cDNA	complementary (copy) deoxyribonucleic acid
cry	cryptochrome (blue light photoreceptor)
CRY	gene encoding for cryptochrome
cv.	cultivar
D	dark
DNA	deoxyribonucleic acid
dNTP	a mixture of the deoxyribonucleic acids
EDTA	ethylenediaminetetraacetic acid
EMS	ethyl methanesulfonate
EOD-FR	end-of-day far-red light response
EtOH	ethanol
FR	far-red light
FR-HIR	far-red high irradiance response
GA_X	gibberellic acid X
GC-MS	gas chromatography-mass spectrometry
GGDP	geranyl geranyl diphosphate
IAA	indole acetic acid
IAA	isoamyl alcohol
IP_3	inositol triphosphate
kDa	kilodalton (protein mass unit)
LD	long day (18 hour daylength)
LFR	low fluence response
MeOH	methanol
MOPS	3-(N-morpholino) propanesulphonic acid
mRNA	messenger ribonucleic acid
NaAc	sodium acetate
nt	nucleotide
ORF	open reading frame
P_r	red-light absorbing form of phytochrome
P_{fr}	far-red light absorbing form of phytochrome
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PHY	phytochrome apoprotein

PHY	WT gene coding for phytochrome
phy	mutant gene coding for phytochrome
phyA	mutant gene coding for phytochrome A
PHYA	phytochrome apoprotein of phytochrome A
phyA	holoprotein of phytochrome A
R	red light
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SD	short day (8 hour daylength)
SDS	sodium dodecyl sulfate
SIM	selected iron monitoring
TAE	tris acetate EDTA
Ub	ubiquitin
UTR	untranscribed region
UV	ultra-violet light
VLFR	very low fluence response
W	white light
WT	wild type

Chapter 1. General introduction

1.1 The use of garden pea as a model species

Since the seminal work of the Augustinian monk Gregor Mendel (1866) on the particulate nature of inheritance, the garden pea (*Pisum sativum* L.) has been widely used as an experimental system for studies in plant genetics and physiology. Pea is a monocarpic, caulescent (clearly defined nodes and internodes) plant, with compound leaves showing heteroblastic development. Peas have an indeterminate growth habit, with the earliest nodes strictly vegetative and sometimes branching. Later nodes bear the reproductive structures, with one to two flowers typically developing at each reproductive node. The number of seeds which form in each pod is highly variable, changing with both genetic and environmental factors. Peas have cleistogamous flowers and are self-fertilising, allowing the development of genetically stable, true-breeding lines. It is also possible to carry out controlled cross-pollination between lines, by simple emasculation of the mother flower followed by dusting of the receptive stigma with pollen from the chosen donor plant.

The generation span of pea, from planting of the seed to desiccation of the next generation of seeds on the mature plant, is a period of only 3 - 4 months, facilitating rapid breeding programs. Plants are conveniently and easily grown in a glasshouse. The vegetative, floral and seed structures are all of a size compatible with morphological studies and easy manipulation in laboratory procedures. There is a wealth of genetic variability in clear morphological characters which can be easily scored (Ellis 1993), presumably one of the reasons Mendel selected this species for his initial studies of inheritance. Peas are also amenable to grafting, a valuable technique for physiological studies of mobile factors; the suitability of peas to this technique is a distinct advantage over some other model plant species such as the widely used dog-eared mustard cress, *Arabidopsis thaliana* (L.) Heynh. For example, grafting studies have been used to demonstrate the presence in pea of both a mobile floral inhibitor and a floral promoter (reviewed in Reid et al. 1996; Weller et al. 1997a). Furthermore, the size of pea plants means they are suitable for measuring endogenous hormone content on a tissue- and organ-specific basis, from either green or etiolated plants. This is something that cannot be easily achieved with *Arabidopsis* due to the sheer number of plants that would have to be grown to generate sufficient plant material for successful hormone extraction. This benefit of peas as a model system has

been put to use in elucidating the role of gibberellins in controlling internode elongation (reviewed in Ross 1994). Finally, the duration of time over which pea has been a chosen model species for experimentation has lead to the generation of large collections of genetic stocks and well-established linkage maps (Weeden et al. 1996, Koornneef et al. 1997).

There is also the potential for agricultural and economic gains to arise out of studies of pea. Peas are an important high-protein crop supplying both human food and animal feed. This crop has a long history, with Smartt (1990) tracing cultivation of peas to as early as 7000 - 6000 BC. In 1997 the world production of dried peas alone was estimated to be 16 million tons (Cousin 1997), which clearly represents a significant economic investment. Peas constitute approximately the world's fourth most important grain legume crop, behind soyabean, ground nuts and bean (Davies 1993). One benefit of the crop is its ability to yield well in the absence of nitrogenous fertilisers, making it a valuable contributor to sustainable agriculture systems (Davies 1993). Yield is variable and all-important, such that there is certainly scope for an increased understanding of growth processes such as seed development and flowering control at a genetic level to be applied to improving yields and qualities of future crops. Studies of crop yield indicate that vigorous vegetative growth discourages partitioning of resources into seeds (e.g. Cousin et al. 1985), and by introducing single gene mutations which act to reduce plant height and leaf area, and increase branching, yield may be increased (Cousin 1997).

At the molecular level, pea is not such an ideal model species. The usual species of choice for molecular genetic studies aimed at linking DNA to phenotype is the crucifer *Arabidopsis thaliana*. This plant is small (commonly grown in petri dishes), has a very short generation time, a small genome (7×10^7 bp, Alberts et al. 1994), and is easily transformed (see Koornneef et al.1997). A successful project is underway to completely sequence the *Arabidopsis* genome (10th International Conference on *Arabidopsis* Research, Australia 1999) and this is rapidly nearing completion. In contrast, the larger genome of pea (around 3.8 - 4.8×10^9 bp per haploid genome, in the same order of magnitude as the human genome [Ellis 1993]) makes some molecular approaches difficult in this species, and although transformation protocols have been developed in some laboratories (e.g. Grant et al. 1995, Bean et al. 1997), they are not yet universally successful or applicable.

However, there remains general recognition in the field that the amount of information that can be gained from physiological and biochemical studies of pea validates its continued use as a complementary model species to others such as *Arabidopsis*, and compensates for some of the difficulties encountered in studies at the molecular level (Murfet and Reid 1993, Koornneef et al. 1997). Well-characterised pea mutants in flowering, phytochrome and gibberellin pathways can and do add significantly to the knowledge gained from *Arabidopsis* and a range of other model species, including tomato, rice, maize, petunia and *Antirrhinum* (Koornneef et al. 1997). Maintaining a range of model species as the focus for experimentation will help to ensure that valid generalisations can be drawn relating to processes of growth and development across the plant kingdom. Establishing gene homologies at the molecular level will allow researchers to capitalise on the advantages of each model species for different types of experimentation (Murfet and Reid 1993), and then to synthesise the information obtained into a cohesive model of developmental processes.

1.2 The use of mutants in dissecting plant developmental processes

The study of developmental mutants has the potential to provide insight into the regulatory mechanisms controlling plant growth and development. The co-ordination of plant development is dependent on interactions between environmental factors such as light and temperature, and internal factors such as endogenous plant hormones and light photoreceptors and their underlying genetic controls.

Whether they have arisen spontaneously or been generated through mutagenesis and screening programs, single-gene mutants have already proved invaluable in forming an understanding of the interaction of factors involved in the overall regulation of developmental programs. For example, the characterisation of *Arabidopsis* ethylene mutants, and an analysis of epistatic interactions among them, has helped to delineate the signal transduction pathway for ethylene-responses following hormone-receptor binding in this species (reviewed in Ecker 1995). Study of internode mutants in *Pisum sativum* and other species has resulted in definition of a role for endogenous gibberellins in the control of stem elongation, and also provided insight into the GA biosynthetic pathway (Ross 1994, Hedden 1999). Beveridge (2000) summarises the use of single-gene mutants of pea to analyse the role of auxins and cytokinins in controlling branching patterns. At least 11 major loci are known to interact to control the flowering phenotype of pea (reviewed in Murfet and Reid 1993, Reid and Howell

1995, Reid et al.1996, Weller et al. 1997b) and the roles of these genes have been defined by careful study of mutant phenotypes under a range of environmental conditions.

This selection of examples illustrates the progress towards understanding development that can be made through investigation of mutants at the morphological and physiological levels. In combination with molecular biology techniques, specific genes involved in developmental processes can be identified and characterised.

1.3 The use of pea mutants to study the phytochrome system

The light incident on a growing plant may vary in quality, quantity, direction and periodicity and constitutes one of the primary environmental cues available to these sessile organisms. The regulation of plant development by the light environment is referred to as photomorphogenesis (Kendrick and Kronenberg 1994) and is mediated by a group of photoreceptors that sense various parts of the light spectrum. Amongst these are the phytochromes, which sense light in the Red (R) and Far-Red (FR) regions of the light spectrum (Vince-Prue 1994), and have been widely investigated. The majority of studies have utilised *Arabidopsis*, although tomato, pea and cucumber, tobacco and rice are among the other model species that have received some attention in this field. In *Arabidopsis* a family of five phytochromes - A, B, C, D and E - has been identified (Sharrock and Quail 1989). These genes have subsequently been cloned (Sharrock and Quail 1989, Clack et al. 1994), and studies of chromophore- and apoprotein-deficient mutants and transgenic plants have helped in the assignation of physiological roles to the various family members (reviewed in numerous places including Kendrick and Kronenberg 1994; Quail et al. 1995, Quail 1998).

Although phytochrome chromophore-deficient and apoprotein mutants have already been studied in *Arabidopsis* (e.g. see Whitelam et al. 1998) and tomato (e.g. see Kendrick et al. 1997), there are sufficient differences in growth responses and experimental advantages to warrant further study of the phytochrome system in pea. Also, peas are agronomically important in their own right, and can be considered representative of a wide-range of related legume crops.

Unlike *Arabidopsis*, which has a basal rosette of leaves, peas are cauline in growth habit. Peas are hypogeal, while *Arabidopsis* plants are epigeal. Peas are also of a sufficient size for biochemical and grafting studies. The seeds are relatively large,

and seedlings can grow for extended periods in darkness producing large amounts of etiolated tissue. Although tomato shares some of these advantages, peas have the additional benefit of being long-day plants with a distinct photoperiodic response. This offers a valuable opportunity to study the interaction between phytochrome and flowering pathways. Mutants of pea can also be used to investigate the possible roles of hormones such as gibberellins and brassinosteroids in light-regulated development (e.g. see Kamiya and Garcia-Martinez 1999). Work with *Arabidopsis* has postulated links between the de-etiolation process and brassinosteroids, and between de-etiolation and gibberellins. Although *Arabidopsis* is suited to in-depth molecular studies, direct measurement of hormone levels has not been feasible and so these postulated links remain speculative. There is potential, following conclusive identification of phytochrome mutants in pea at the molecular level, to refine these speculations with measured hormone data.

There is also a long history of using peas in studies of phytochrome, subsequent to the first identification of this pigment (Butler et al. 1959). Notably, pea-specific monoclonal antibodies have been raised to two types of pea phytochrome (Nagatani et al. 1984) which were then further characterised through immunochemical studies (Abe et al. 1985, 1989). The *PHYA* gene of pea has been cloned (Tomizawa et al. 1986) and sequenced (Sato 1988), and more recently a *PHYB* gene has been identified at the molecular level (N. Beauchamp, pers. comm.). Pea has proven amenable to isolation of mutants apparently lying within the phytochrome pathways. Mutants selectively deficient in phytochrome A (*fun1*; Weller et al. 1997a), phytochrome B (*lv*; Weller et al. 1995) and the phytochrome chromophore (*pcd1*, *pcd2*; Weller et al. 1996, Weller et al. 1997c) have been identified through physiological and biochemical studies. These mutants remain to be characterised at the molecular level.

1.4 Aims and scope of this thesis

The major aim of this thesis was to draw together previously completed physiological studies of phytochrome A mutants of pea (Weller 1996) with molecular characterisation of the mutants, in order to establish a firm link between DNA and phenotype.

Chapter 2 is a review of what is currently known about the molecular nature of phytochromes; structure, functional domains, behaviour as light-regulated protein kinases, and theories relating the localisation of phytochrome within the cell to its

activity. **Chapter 3** briefly outlines methods, plant materials and growth conditions used throughout the thesis. **Chapter 4** presents a molecular characterisation of pea mutants identified as being deficient in phytochrome A, while **Chapter 5** reports an investigation of the molecular nature of a mutant showing exaggerated phytochrome A-like responses. In **Chapter 6** the ability of the phytochrome mutants to green after FR-exposure is examined. **Chapter 7** concludes this section of work with a brief summary of the current knowledge of the phytochrome family in pea, the contributions of this thesis, and suggestions of directions for future work on this system.

In **Chapter 8**, an analysis of the levels of abscisic acid (ABA) present in developing seeds of the gibberellin-deficient mutant *lh-2*, and the effects of ABA on seed development, is presented. This work provides another example of the use of a single-gene mutant of pea in dissecting a developmental process, and of the amenability of pea as a model for seeking to understand the roles of endogenous hormones in development.

Chapter 2. The molecular nature of phytochromes

2.1 Introduction

In darkness a higher plant grows heterotrophically, dependent on preformed seed reserves for energy while striving to reach light. In species such as the experimental model plant *Arabidopsis*, growth form in darkness is characterised by an elongated hypocotyl, an apical hook, and unopened cotyledons. Light triggers de-etiolation, a change in growth form to optimise the seedling for efficient photosynthetic growth. At this stage, hypocotyl elongation is repressed, the apical hook unbends, the cotyledons expand, chlorophyll is synthesised and new gene expression patterns are invoked (McNellis and Deng 1995, Fankhauser and Chory 1997). This suite of morphological and biochemical changes is set in train by the change in the growing plant's environment. As plants occupy a fixed position in space, it is critical they monitor their environment to optimise development under the prevailing conditions. The light incident on a growing plant constitutes one of the primary environmental cues available to a sessile organism, and may vary in spectral quality (wavelength), quantity (fluence), intensity (fluence rate), direction and duration (photoperiod). The ability to detect and respond to changes in these parameters confers adaptive capacity on the plant, and this regulation of plant development by the light environment is referred to as photomorphogenesis (Kendrick and Kronenberg 1994).

Photomorphogenesis is mediated by a group of photoreceptors which sense various parts of the light spectrum; the phytochromes which sense light in the red (R, approx. 600 – 700 nm) and far-red (FR, approx. 700 - 760 nm) wavelengths (Vince-Prue 1994), the cryptochromes which detect blue (B) light / UV-A (320 - 500 nm), and the UV-B receptors (280 – 320 nm)(Senger and Schmidt 1994). Together these photoreceptors can be regarded as the mechanism underlying plant vision (Ahmad 1999). Of these photoreceptors, the phytochromes are the best characterised, and form the focus of this review.

In co-ordination with the other classes of photoreceptors, phytochromes act in the plant as a generally distributed antenna for light. Although plant responses to light are most evident, morphologically, in the seedling making the transition from skotomorphogenic to photomorphogenic growth form (McNellis and Deng 1995), they do continue throughout the life of the plant, from seed germination through to flowering and senescence. The phytochromes have roles in regulating

germination, chlorophyll production, leaf expansion and development, stem elongation, plant architecture, and floral initiation. Phytochromes are thus considered essential regulators for major transitions in plant development. In an ecological context, they are important modulators of vegetative development and exert influence on gravitropism, phototropism and the shade avoidance response (Smith 1995, Parks et al. 1996, Robson and Smith 1997). They are also involved in the entrainment of circadian rhythms (Vince-Prue 1994, Ahmad 1999).

Light control of seedling development has been widely reviewed (Deng 1994, Kendrick and Kronenberg 1994, McNellis and Deng 1995, Pratt 1995, Quail et al. 1995, von Arnim and Deng 1996, Chory 1997, Fankhauser and Chory 1997, Batschauer 1998, Whitelam and Devlin 1998, Whitelam et al. 1998, Ahmad 1999, Deng and Quail 1999), as befits such a fundamental aspect of plant development. Although much has been written about proposed physiological and ecological roles of phytochrome (Kendrick and Kronenberg 1994, Smith 1995), and in more recent times details of the molecular properties of phytochrome have been forthcoming (reviewed in Furuya and Schäfer 1996, Quail 1997), an enduring question of phytochrome research has been - what is the molecular mechanism of action of phytochrome? This has been addressed by many modes of research, with an increasing focus on the use of mutants and transgenic plants to understand the phytochrome signalling process. Analysis suggests that following light perception a complex network of both separate and shared signalling pathway components interact and converge to regulate developmentally relevant genes via a range of master regulators (reviewed in Deng and Quail 1999). Genetic dissection of light-influenced processes implicates the involvement of in excess of 50 loci in the light-signalling network controlling plant development (Fankhauser and Chory 1997).

2.2 The discovery of phytochrome

The discovery of phytochrome and the ensuing history of phytochrome research have been comprehensively documented by Sage (1992). The initial work stemmed from a USDA research program seeking to understand photoperiodism in plants, and the fundamental realisation that light period is a logical and predictive environmental cue, with change in daylength preceding change of the seasons. Daylength has been described as "the only consistently rhythmic feature of the external environment" (Garner and Allard 1923, in Sage 1992), unlike other factors such as temperature, rainfall and light intensity. The search for the biological molecule underlying a plant's ability to sense daylength lead to the

eventual identification and characterisation of phytochrome as the photoreceptor responsible for R/FR reversible reactions of plants (Butler et al. 1959). Phytochrome was thus the first plant photoreceptor to be identified and later purified. As well as being present in higher plants (Quail et al. 1995), phytochromes have been identified in mosses, ferns and green algae (Pratt 1995).

2.3 Evidence of multiple phytochromes within a single plant

Difficulties in correlating the results of physiological experiments with the levels of phytochrome measured spectrophotometrically lead to the first suspicions that there may be more than one phytochrome type, or pool, within a plant (Kendrick and Kronenberg 1994). It appeared to researchers that there may be both a bulk pool of phytochrome detectable by spectrophotometry yet biologically inactive; and a second, minor but active pool (reviewed in Pratt 1995). The apparent variation in properties of different pools of phytochrome within the same plant, for example the identification of both light labile and light stable pools, added to the evidence suggesting multiple phytochromes. A third factor is the wide range of sensory activity and response seen in plants across the R/FR portion of the light spectrum. These include the so-called very low fluence responses (VLFR); low fluence responses (LFR) characterised by R/FR reversibility; FR-mediated high irradiance responses (FR-HIR), and responses to FR light given as an extension at the end of day (EOD-FR) (see Mancinelli 1994).

The first compelling physical evidence of multiple phytochromes was the isolation of immunologically distinct phytochromes from pea (Abe et al. 1985) and oat (Hilton and Thomas 1985). This was followed by the critical demonstration that the two types of phytochrome found in pea differed in the amino acid sequence of the apoprotein portion of the molecule (Abe et al. 1989), indicating them to be the products of distinct genes. This implied the presence of a gene family of phytochromes. Molecular confirmation was provided by the identification (Sharrock and Quail 1989) and cloning (Sharrock and Quail 1989, Clack et al. 1994) of five members of the phytochrome gene family in *Arabidopsis*, *PHYA*, *B*, *C*, *D* and *E*.

2.4 Physiological roles and response modes of phytochromes

The selection and characterisation of mutants deficient in responses to selective portions of the light spectrum has allowed the assignation of both distinct and overlapping roles to some members of the phytochrome gene family (Reed et al.

1994, Furuya and Schäfer 1996, Whitelam and Devlin 1997). The majority of these mutant-based studies have utilised *Arabidopsis* as a model species, although tomato, pea and cucumber mutants have also been reported.

There is general acceptance that light-labile phytochrome A is the main photoreceptor responsible for perception of FR light, mediating both the so-called high-irradiance response (HIR) and also very low fluence responses (VLFR) (Quail et al. 1995). Phytochrome A has a partial role in the de-etiolation process of seedlings making the transition from skotomorphogenic (light-seeking) to photomorphogenic (light-utilising) growth habit. Phytochrome A-deficient plants fail to de-etiolate when grown under monochromatic FR light, but under W they de-etiolate normally, indicating that phytochrome A is not essential to seedling growth in the normal white light environment (Nagatani et al. 1993, Parks and Quail 1993, Whitelam et al. 1993, Weller et al. 1997a). However, phytochrome A-deficient mutants of *Arabidopsis* and pea have been reported to lack the ability to detect daylength extensions (Johnson et al. 1994, Jackson and Thomas 1997, Weller et al. 1997a), demonstrating some role for phytochrome A in development of the mature plant.

Light-stable phytochrome B mediates the R/FR reversible low fluence responses (Reed et al. 1994). Since phytochrome B monitors R and the ratio of R/FR, in the absence of this phytochrome plants cannot detect or avoid shading and neighbour proximity (Quail 1994b). Phytochromes A and B exhibit complementary sensitivities to R and FR light, and exert antagonistic effects on hypocotyl elongation in varying light environments (Quail et al. 1995). The two phytochrome types share a common chromophore but differ in stability, immunochemical and spectral properties, proportions present in the plant at various developmental stages and under varying environmental conditions, and the contributions they make to the regulation of different physiological responses (Mancinelli 1994).

Less is known about the roles of the other members of the phytochrome gene family. This is due to their relatively recent identification, the limited range of species in which they have been demonstrated to occur, and the very few mutants identified with specific deficiencies in these phytochromes. The difficulty in detecting mutants may be a result of inappropriate screening, lethality, or more likely functional redundancy between members of the phytochrome family. A naturally-occurring deletion in the *PHYD* gene of the *Arabidopsis* Wassilewskija ecotype (Aukerman et al. 1997) has facilitated definition of a role for

phytochrome D. This phytochrome, which shares about 80% sequence identity at the amino acid level with phytochrome B, acts in conjunction with phytochrome B in regulating shade avoidance responses but may not be an essential phytochrome in some natural environments (Aukerman et al. 1997, Devlin et al. 1999). A mutagenesis program has resulted in the identification of a single phytochrome E mutant; phenotypic study indicates that phytochrome E influences both internode elongation and flowering time in *Arabidopsis* (Devlin et al. 1998). Phytochrome C is postulated to enhance leaf expansion in *Arabidopsis* (Qin et al. 1997), although this is based on overexpression of phytochrome in transgenic plants, as no phytochrome C mutants have been identified to date.

2.5 Physical properties of phytochrome

Phytochrome is a light-activated molecular switch which exists in two interconvertible forms, one absorbing R (Pr) and the other absorbing FR (Pfr) light, in an equilibrium influenced by the spectral make up of the available light. The light-sensing capacity resides in an open-chain tetrapyrrole chromophore which in effect measures the ratio of R/FR light incident on the plant. The chromophore molecule is phytochromobilin, related to bilin pigments of cyanobacteria and red algae (reviewed in Hughes and Lamparter 1999) and derived from haem precursors (Björn 1994). The absorption bands of the two phytochrome forms are broad and overlapping, such that a photoequilibrium is reached even under monochromatic light (Björn 1994). The two forms of phytochrome do have distinct spectral properties, due to a change in the electronic state of the chromophore molecule and/or due to conformational change of the protein pocket in which the chromophore is situated (Björn 1994). Conversion of Pr to Pfr involves a Z to E isomerization of the C15 double bond between the C and D tetrapyrrole rings (reviewed in Braslavsky et al. 1997). P_{fr} is believed to be the physiologically active form of phytochrome (Mancinelli 1994).

In plant extracts the phytochrome molecule exists as a heterodimer of 120 - 130 kDa. The chromophore moiety attaches to a cysteine of the apoprotein (approximately residue 321) via a covalent linkage to form a holophytochrome monomer, which then pairs with a second to occur as a dimer (Furuya and Song 1994). The PHY apoprotein and the chromophore lack photoreversible functions and bioactivity until they are assembled to form holophytochrome. Phytochrome synthesis does not appear to be limited by chromophore availability, as evidenced by the ability of plants overexpressing heterologous phytochromes to synthesise and assemble functional heterologous phytochrome *in vivo* in addition to their

normal complement of native phytochrome (Cherry and Vierstra 1994). Phytochrome is synthesised as Pr; the chromophore is exported from the plastids and assembles with the apoprotein in the cytoplasm (Furuya and Song 1994). Light input causes interconversion between forms, setting in train the Pr/Pfr equilibrium. Once formed, Pfr either undergoes dark reversion to Pr (this only occurs for some phytochrome types in some species); undergoes form-specific degradation, possibly mediated through recognition of sequestered ubiquitin-phytochrome conjugates; or interacts with unknown reaction partner(s) X to trigger a cascade of signalling ultimately leading to physiological response (Mancinelli 1994).

The primary structure of phytochrome is defined by the RNA/cDNA sequence (see section **2.7**), and is comparable to other well-known soluble globular proteins in terms of amino acid composition and mean hydropathy index (Furuya and Song 1994). Some aspects of secondary structure have been revealed by circular dichroism (CD) studies, the only practical method yet devised for studying such a large protein in solution. The secondary structure is thought to be, at least in part, dependent on the chromophore interaction with the protein chain. Solubility differences between the apoprotein and the holophytochrome would tend to support this (Furuya and Song 1994). Circular dichroism (CD) studies of *Avena* (oat) report regions of extensive α-helical folding and a marked absence of ß-sheet in native holoproteins. In contrast, CD-based investigation of pea phytochrome revealed significant amounts of ß-sheet (reviewed in Furuya and Song 1994).

The tertiary and quaternary/dimeric structure of phytochromes is evident through electron microscopy and small-angle X-ray scattering analysis (Furuya and Song 1994). Electron microscopy suggests phytochrome dimers have a Y-shaped, tripartite structure. Dimerisation contact is believed to occur via the carboxy-terminal domain of monomer units (Chory 1997). Deletion analysis indicates residues terminal of residue 750 are required for this interaction. In pea phytochrome, Phe 744 and Gln 1128 have been identified as individual residues involved in dimerization (Yamamoto and Tokutomi 1989). Any more precise determination of the attachment and conformation of the chromophore moiety within native phytochrome will require crystallographic analysis (Furuya and Song 1994). It is thought, however, that the chromophore is more exposed in the Pfr form than in the Pr form, and that this exposure of acidic amino acid residues to the surface results in a higher net negative charge on the Pfr form of phytochrome (Furuya and Song 1994).

2.6 The phytochrome gene family

Direct molecular evidence of a phytochrome gene family came from the isolation, cloning and sequencing of the family members in *Arabidopsis* (Sharrock and Quail 1989, Clack et al. 1994). *PHYA* genes had earlier been cloned from oat (Hershey et al. 1985, 1987; Grimm et al. 1988), zucchini (Sharrock et al. 1986), pea (Sato 1988), maize (Christensen and Quail 1989) and rice (Kay et al. 1989a) - and since from a whole range of species. As of March 2000 there are in excess of 115 *PHYA* or *PHYA*-like sequences entered in GenBank (www.ncbi.nlm.nih.gov), and a total of more than 500 phytochrome or phytochrome-like sequences. Multiple and distinct phytochrome species, the products of a divergent gene family, have been identified in all higher plant species examined to date (Mathews and Sharrock 1997).

Before the phytochromes were identified at the gene level, two broad types were recognised. Type I was described as a light labile phytochrome, abundant in etiolated tissue but rapidly degraded as the Pfr form following exposure to light. Type I phytochrome is now considered to be encoded by *PHYA*-type genes. In contrast, Type II phytochrome occurs at low levels in plant tissues but is relatively stable as Pfr and in many species becomes predominant (albeit at low levels) in green tissue due to the selective depletion of Type I. Type II phytochromes are encoded by *PHYB*, *PHYC* and possibly the remaining members of the gene family identified so far (Quail 1994a).

The presence of a family of divergent and differentially regulated phytochrome genes leads to the obvious question of whether each family member has a discrete sensory function and individual action mechanism. In aiming to answer this question, intensive study of the gene family has been undertaken. The five *Arabidopsis* phytochrome genes exist as single copies within the genome (Sharrock and Quail 1989). As *Arabidopsis* has one of the smallest genomes among higher plants, this might imply the existence of at least as many phytochrome family members in other plant species. Alternatively, other species may contain distinct forms and variable numbers of phytochromes depending on the timing of their evolutionary divergence from the *Arabidopsis* lineage.

The five *Arabidopsis* phytochrome genes display substantial variation between each other. When subjected to pairwise comparisons, the amino acid sequences show only 46 - 56 % identity (Sharrock and Quail 1989, Clack et al. 1994), with the exception of *PHYB* and *PHYD* which show approximately 80 % identity

(Clack et al. 1994). The five phytochromes can therefore be assigned to four subfamilies: *PHYA*, *PHYB/D*, *PHYC* and *PHYE* (Pratt 1995). Although it is probable that all members of the gene family present in *Arabidopsis* have now been identified, through the use of degenerate PCR protocols and low-stringency Southern hybridisation, this cannot be concluded for sure until sequencing of the *Arabidopsis* genome is complete. The size of the phytochrome family in other less extensively studied species remains to be defined.

Intensive studies of the tomato genome (Pratt 1995; Hauser et al. 1995) have resulted in the determination of five phytochrome sequences – *PHYA*, *PHYB1*, *PHYB2*, *PHYE* and *PHYF*. *PHYF* is thought to define a new subfamily, not represented in *Arabidopsis* (Hauser et al. 1995), although there is some suggestion that *PHYF* should be classified as a member of the *PHYC* subfamily (Mathews and Sharrock 1997). In sorghum three phytochromes (*PHYA*, *PHYB* and *PHYC*) have been identified from PCR fragments, and low stringency Southern hybridisation suggests the presence of further phytochromes in the sorghum genome (Pratt 1995). Multiple *PHYB*-types have been suggested to occur in *Daucus* (Mathews et al. 1995), tobacco and potato (Kern et al. 1993). Howe et al. (1998) have presented evidence for the presence of one *PHYA* and two *PHYB* loci in black cottonwood, but were unable to detect representatives of the *PHYC/F* and *PHYE* subfamilies. In pea to date a single-copy *PHYA* gene (Sato 1988), a *PHYA*-pseudogene (Sato 1990) and a *PHYB* gene (N. Beauchamp, pers. comm.) have been sequenced. The variety of family members observed in these species highlights the need to continue to study this gene family in a wider range of species, in order to be certain of identifying a full complement of phytochromes.

The occurrence of phytochromes and phytochrome-like proteins throughout the plant kingdom renders them useful tools in the study of molecular evolution. Kolukisaoglu et al. (1995) analysed 32 partial sequences and 12 full-length sequences and determined that the divergence of the phytochrome gene family predates the evolution of Angiosperms, as distinct groups are identifiable within the lower plants. There is some indication from their study that phytochrome gene duplication giving rise to *A*, *B*, and C-types occurred within the seed ferns. Such molecular-based studies may give clear indications of the evolution of land plants and, in conjunction with fossil data, can be used to understand the history of the development of the modern flora. Hence phytochrome sequences provide information not only about the phylogeny of this multigene family, but also broader insights into land plant evolution.

Mathews and Sharrock (1997) identify the phytochrome of the green alga *Mesotaenium* (Lagarias et al. 1995) as the most ancestral type yet sequenced. Their analysis puts the duplication event leading to divergence of phytochromes A/C from phytochromes B/D/E near the origin of seed plants, and further duplications within the two subgroups near the origin of the flowering plants. Hence the complexity of the phytochrome family may have increased along with the evolution of novel land plant morphologies (Mathews and Sharrock 1997).

2.7 Structure and expression of the phytochrome genes

Comparison of the mRNA for the various phytochrome genes reveals a conserved structure across the family and across plant species. The genes generally contain five to six exons, and four or five introns, with one in the 5' untranslated region (UTR) and one apparently confined to the 3' UTR of monocot sequences (Quail 1994a). Another conspicuous difference between monocot and dicot phytochrome sequences is that monocot genes have only one identified transcription start site, whereas sequences from the dicots pea, *Arabidopsis* and tobacco reveal 3 distinct transcription start sites (Sato 1988, Dehesh et al. 1994, Adam et al. 1995), generating transcripts of variable length and differentially regulated expression (Tomizawa et al. 1989, Adam et al. 1995, Cantón and Quail 1999).

The approximately 125 kDa mature phytochrome apoprotein consists of about 1100 amino acids, and can be considered as a 70 kDa amino-terminal domain fused to a 55 kDa carboxy-terminal domain by a proteolytically-susceptible linker region (Quail 1994a). The site of chromophore attachment is at a completely conserved cysteine moiety (position 374 acccording to the numbering system of Quail 1997, derived from the consensus sequence of Mathews et al. 1995). The amino-terminal of the molecule is approximately equivalent to the sequence of the major 5' exon, while the sequence encoding the carboxy-terminal domain contains about 3 separate exons which may represent distinct structural or functional sub-domains (Quail 1994a).

In *Arabidopsis* phytochrome A is abundant in darkness but decreases in continuous light conditions. Phyotchromes B and C are of low abundance but occur at relatively constant levels, with no significant regulation by light. These trends are matched at both the protein and mRNA levels (Quail 1994a). The decrease in phytochrome A levels in light is due both to degradation of the Pfr form, and light-induced repression of *PHYA* gene expression. The question of

whether photoregulation of *PHYA* mRNA is occurring at the transcriptional or post-transcriptional level is addressed by the work of Komeda et al. (1991) with transgenic petunia. Promoter-GUS fusions created with the pea *PHYA* promoter reveal the spatial organisation of expression, with high expression in the apex, declining along a gradient down the hypocotyl, and barely detectable in the root. Importantly, light exposure caused down-regulation of GUS expression, indicating that - at least in part - negative regulation of the expression levels of the pea *PHYA* gene occurs at the transcriptional level (i.e. the light-interacting element is contained within the pea *PHYA* promoter).

2.8 Domain mapping using mutants and transgenic plants

The mapping of various molecular domains of phytochrome, often by selectively deleting them or swapping them between the various phytochromes, has been used to gain information about the biochemical mechanisms underlying phytochrome action, and to define domains necessary and/or sufficient to confer certain properties and responses of phytochrome (Chory 1997, Quail 1997). Comparison of phytochrome sequences with other sequences of known function has also offered insights into the mechanisms at work and will be considered separately (see section **2.9**). Reverse and forward genetics have also played a role, including the characterisation of mutants at the molecular level, phytochrome overexpression studies, expression of antisense sequences to reduce expression, and the identification of dominant-negative interactions between native and heterologous phytochromes (Cherry and Vierstra 1994).

Quail (1997) has presented a summary figure of the known domains and activities of a generalised phytochrome molecule; an adaptation of this is shown in Fig. 2.1. All position numbers cited are based on those given in this figure.

Structural domains

The phytochrome peptide folds into two well-defined domains. The chromophore is covalently attached to the amino-terminal, tucked into a hydrophobic pocket of the globular domain. In contrast the carboxy terminal is a more linear, extended fragment; the two domains are connected by a short linker segment which is vulnerable to proteolysis. This junction occurs at around amino acid position number 673 (based on the consensus sequence numbering adopted by Mathews et al. 1995 and developed from the extremities of *Arabidopsis* phytochrome B which is the longest known family member; see Quail 1997). The linker region is approximately 150 amino acids in length and (towards the carboxy-terminal side)

appears to be a "hot spot" for missense mutations affecting phytochrome A and phytochrome B function (Wagner and Quail 1995, Xu et al. 1995, Bradley et al. 1996). In this region there are two direct repeats of 40 amino acids in length, themselves separated by a linker. These repeats show some homology to PAS domains implicated in protein-protein interactions between basic helix-loop-helix/PAS transcription factors (Lagarias et al. 1995; reviewed in Hughes and Lamparter 1999).

Spectral activity and integrity

The spectral activity of phytochrome is reliant on correct assembly of apoprotein and chromophore to form holophytochrome. The molecular determinants responsible for catalysing chromophore attachment lie in the amino-terminal of the apoprotein, somewhere between positions 115 - 450 (Boylan and Quail 1991, Deforce et al. 1991, Cherry et al. 1993, Cherry and Vierstra 1994, Wagner et al. 1996). Detailed site-specific mutational analysis of five conserved residues surrounding the cysteine at position 374 to which the chromophore actually attaches has failed to reveal any that are essential to chromophore lyase activity (Deforce et al. 1993, Song et al. 1997).

A much more extensive region of sequence is necessary to maintain spectral activity equivalent to that of full-length phytochrome. Most, and possibly all, of the amino-terminal domain is required. In its absence the phytochrome molecule loses its capacity for R/FR reversibility and the absorption spectra of Pr and Pfr phytochrome forms differ from those of native phytochrome (Quail 1997). Deletion analyses in transgenic plants have indicated that the entire amino-terminal (positions 1 - 673) is sufficient for spectral activity (Boylan and Quail 1991, Cherry et al. 1993, Boylan et al. 1994). Subdomains required for stability and integrity of the Pfr form (51 - 94), and both the Pfr and Pr forms (51 - 115) have been also been identified (Cherry et al. 1992, 1993, Boylan et al. 1994, Jordan et al. 1995a). Some further residues towards the carboxy end of the amino-terminal (524 - 673) are also considered necessary to normal spectral function of the phytochrome molecule (Boylan and Quail 1991, Cherry et al. 1993, Boylan et al. 1994).

Dimerization

Proteolytic studies and the use of antibodies to map epitopes have provided evidence that the carboxy-terminal domain carries the molecular determinants for dimerization (Furuya and Song 1994, Chory 1997, Quail 1997). Contrastingly, the amino-terminal domain exists as a monomer following treatment with

cleavage agents (Jones and Quail 1986). Amino-terminal domains expressed in transgenic plants also act as globular monomers, confirming the indication that they do not carry dimerization determinants (Cherry et al. 1993, Boylan et al. 1994, Wagner et al. 1996); whereas transgenic expression of the carboxy-terminal domain of phytochrome B leads to formation of homodimers in *Arabidopsis* (Wagner et al. 1996). Removal of amino acids after position 981 results in loss of ability to dimerize (Cherry et al. 1993), with some indication that the critical residues in fact lie between positions 1116 and 1161 (Edgerton and Jones 1992, Cherry et al. 1993).

Pfr degradation

Conversion of phytochrome A to the Pfr form is followed rapidly by intracellular degradation of phytochrome A. This light-lability is not seen for phytochromes B, C, D and E which are therefore considered to be "light stable". Domain swapping experiments dependent on expression of manipulated transgenes provide some indication that the molecular determinants specifying degradation of Pfr phytochrome A lie within the amino-terminal domain (Wagner et al. 1996). However, a contiguous carboxy-terminal is required, as when expressed alone the amino-terminal is light-stable *in vivo* (Boylan et al. 1994). Deletion of the extreme end of the carboxy-terminal appears to stabilise the phytochrome A polypeptide as Pfr, suggesting the localisation of one of the degradation determinants at this end of the molecule (Vierstra 1996). As the carboxy-terminal domain of either phytochrome A or phytochrome B is able to induce degradation when fused to the phytochrome A amino-terminal, this determinant of degradation appears to be common to both phytochrome family members (Wagner et al. 1996).

The mechanism controlling phytochrome degradation is not known for sure, but clearly discriminates in some way between the molecular structure of Pfr and Pr. One persistent theory is that the Pfr is degraded via ubiquitin-dependent proteolysis (Vierstra 1994). There have been numerous isolations of conjugates with one or more ubiquitin molecules joined to phytochrome (Ub-P conjugates), identified on the basis of their large size in SDS-PAGE fractionation. Ub is a highly conserved 76 amino acid protein with a compact globular core which becomes covalently bound to target proteins and is known to form multi-Ub chains, acting as recognition signals for a proteolytic complex (Vierstra 1994). Ub–P appear and disappear concomitantly with Pfr degradation and are not detectable in dark-grown plants. Most of the Ub-P are found in sequestered areas of phytochrome (SAPs), suggesting a role for sequestration in the degradation

process. However, the amount of sequestration and conjugation to Ub is considered insufficient to account for all the Pfr degradation observed, and a direct link between ubiquination of PfrA and degradation has not yet been demonstrated (Quail 1997). Alternatively, there is a potential degradation signal termed the PEST-sequence (Rogers et al. 1986) near the chromophore attachment site in phytochrome A (but absent from phytochrome B, C, D and E sequences). However, at this stage there is a lack of firm data to implicate the PEST sequence in control of Pfr degradation (Quail 1997).

Biological activity

Transgenic expression of deletion derivatives has been used to identify regions of the phytochrome molecule essential to normal biological activity. As a generalisation, it is apparent that removal of short segments from either terminus is sufficient to either eliminate or seriously modify the level of detectable activity of the phytochrome molecule. Determinants within the first 60 residues and the final 35 residues appear to be essential (reviewed in Quail 1997). Internal deletions in the proximity of the linker can also eliminate normal activity (Wagner et al. 1996). There is as yet no way to differentiate whether these deletions eliminate activity by loss of direct involvement in photoreceptor activity, or by causing conformational change in remote parts of the molecule critical to activity.

An intriguing "gain of function" effect has also been identified; deletion of a serine-rich region between positions 42 - 51 (Jordan et al. 1995), or site-directed substitution of all these serines by alanines (Stockhaus et al. 1992), leads to enhanced phytochrome A activity. This indicates that when present these serine residues are somehow involved in dampening the activity of native phytochrome A.

Based on results of reciprocal domain-swapping studies in transgenic plants, the molecular determinants of phytochrome's sensory specificity are believed to reside in the amino-terminal (Quail 1997). Experiments indicate that phytochrome A(amino-terminal)/B(carboxy-terminal) fusions show phytochrome A-like responses, while phytochrome B/A fusions resemble phytochrome B in their activity (Wagner et al. 1996). Deletion of the amino-terminal 52 amino acids of phytochrome A is sufficient to result in loss of FR-perception (but not responsiveness to R), localising one or more determinants of phytochrome A photosensory specificity within this fragment (Boylan et al. 1994).

The amino terminal expressed alone is insufficient for biological activity, despite retaining photoactivity and spectral integrity. A contiguous carboxy-terminal domain is necessary for retention of signal transduction of perceived light signals; phytochrome A and B carboxy-terminals are interchangeable for this function, indicating the existence of common determinants between family members (Wagner et al. 1996). Mutants have been identified which are competent in photoperception but not in signal transduction response. A cluster of such mutations are known at the proximal end of the carboxy-terminal (positions 681 - 838, adjacent to the linker region). In particular, multiple substitutions have been noted to occur at four distinct positions within an 18 residue stretch (positions 776 - 793) (Quail 1994a, Quail et al. 1995, Quail 1997). These data imply that this core region is the site of determinants required for successful communication of perceived light signals to the signal transduction pathway. Since phytochrome A and B carboxy-terminals are functionally interchangeable, and because this class of mutations cluster in the same region of sequence for both phytochrome A and B, it is a reasonable conclusion that the two phytochromes share at least some common signal transduction mechanisms (Quail 1997).

2.9 Insights into phytochrome function from sequence alignments

Multiple alignments assist in the recognition of regions and residues conserved throughout evolution and therefore likely to hold some functional or sensory significance. For instance, in the phytochromes sequenced to date the greatest level of sequence identity is clustered in the region around the chromophore attachment site (Quail 1994a); this is suggestive of strong evolutionary pressure conserving the chromophore environment. Lower levels of identity in the remainder of the protein may be the result of less rigid structural constraints on this portion, or indicate the diversification of elements/determinants involved in photosensory and functional specificity (Quail 1994a).

Sequence information and alignments also facilitate database searches for similar proteins, generating clues about possible action mechanisms of the phytochromes. This approach was instrumental in suggestions that phytochromes may act as light-regulated protein kinases. The source of this hypothesis was the evidence from database searches presented by Schneider-Poetsch et al. (1991) indicating that the distal portion of the carboxy-terminal of phytochrome (amino acid positions 931 - 1210) displays sequence similarity to the sensor histidine kinase module of bacterial two-component signalling systems. The suggestion was thus made that phytochromes may act as a family of histidine kinases, regulated by

signals from the ambient light environment. This conflicted with a previous observation of serine/threonine kinase activity associated with purified preparations of higher plant phytochrome A (Wong et al. 1986).

Quail (1994b) noted at least three distinct arguments against the veracity of this hypothesis: (1) the conserved histidine residue which is the target of bacterial histidine kinases is absent from the majority of higher plant phytochromes, having been identified only in monocot phytochrome As and *Arabidopsis* phytochrome C; (2) there are no reports in the literature of detectable phosphorylation of histidine residues by phytochrome; and (3) site-directed mutagenesis of residues conserved between histidine kinases and phytochromes failed to reduce activity of transgenically-expressed phytochrome.

However, recent sequencing of cyanobacterial genomes has revitalised the hypothesis that phytochromes have inherent kinase activity. A chromatic adaptation sensor has been cloned from the filamentous cyanobacterium *Fremyella diphosiphon* (Kehoe and Grossman 1996). Sequence information indicates this protein is a potential histidine kinase, but bears an amino-terminal extension with some homology to the phytochrome chromophore binding domain (reviewed in Allen and Matthijs 1997). An open reading frame (ORF) identified in *Synechocystis* sp. PCC6803 shows similarity to both phytochromes and sensor histidine kinases (Hughes et al. 1997, Yeh et al. 1997). This ORF may represent a photoregulated histidine kinase; the related plant phytochromes may be the evolutionary descendants of these prokaryotic molecules (Yeh et al. 1997, Yeh and Lagarias 1998). The product of the ORF is able to autocatalytically attach tetrapyrrole chromophores, and form a chromoprotein demonstrating R/FR-red reversibility similar to that seen in plant phytochromes (Hughes et al. 1997). When expressed in a yeast vector, the protein shows histidine kinase activity (Yeh et al. 1997).

Also of interest is a unique phytochrome gene isolated from the moss *Ceratodon purpureus*. Although the carboxy-terminal of conventional phytochromes is homologous to the catalytic domain of bacterial sensor histidine kinases, the carboxy-terminal of the *CpPHY1* gene is homologous to the catalytic domain of eukaryotic serine/threonine/tyrosine kinases (Thümmler et al. 1992).

2.10 The molecular mechanism of action of phytochrome

The central and longlasting aim of phytochrome research has been to identify the general molecular mechanism of action underlying phytochrome activity. A convergence of evidence now indicates that eukaryotic phytochromes are light-regulated protein kinases (Cashmore 1998, Reed 1998, Yeh and Lagarias 1998, Ahmad 1999) - as first suggested by Borthwick and Hendricks (1960).

A series of biochemical studies (e.g. Wong et al. 1986, Wong and Lagarias 1989) presented evidence of phytochrome preparations possessing inherent serine/threonine protein kinase activity. However, since phytochrome does not show clear sequence homology to known serine/threonine/tyrosine kinases and there were some reports of loss of protein kinase activity from phytochrome preparations after extensive purification (Grimm et al. 1989, Kim et al. 1998), there was some doubt as to whether the reports of inherent kinase activity were accurate.

Schneider-Poetsch et al. (1991) advanced the idea that phytochrome may act as a histidine kinase. As described in section **2.9**, sequencing of phytochromes from cyanobacteria boosted this hypothesis. These hypotheses have been drawn together by the work of Yeh and Lagarias (1998) who propose that eukaryotic phytochromes have histidine kinase ancestry but now function as light-regulated serine/threonine protein kinases. They demonstrate that highly purified oat phytochrome A generated in a yeast expression vector possesses serine/threonine kinase activity, undergoes autophosphorylation, and successfully phosphorylates Rcp1 (a response regulator identified as a substrate of the cyanobacterial phytochromes; see Yeh et al. 1997).

The evidence for phytochrome's kinase activity is becoming overwhelming. The next step in elucidating the action mechanism will be the identification of the phosphorylation targets for native phytochrome *in vivo*. Ahmad et al. (1998) have shown the blue-light receptor cryptochrome to be a phosphorylation target for phytochrome *in vitro*. Considered together with genetic evidence from *Arabidopsis* of reduced CRY1 activity in the absence of phytochromes A and B (Ahmad and Cashmore 1997), it is possible that cryptochrome is a co-occurring biological substrate for phytochrome (Ahmad 1999).

A second possible target has been identified in *Arabidopsis* through yeast-two hybrid screening. Phytochrome Interacting Factor 3 (PIF3) interacts with both

phytochromes A and B and is a potential reaction partner for phytochrome *in vivo* (Ni et al. 1998). PIF3 is localised in the nucleus, is a basic helix-loop-helix protein and seems to be involved in phytochrome responses in phytochrome B overexpressing and antisense transgenic plants (Ni et al. 1998, 1999). Whether this translates to an *in vivo* interaction between PIF3 and nuclear-localised phytochrome B (Sakamoto and Nagatani 1996), and whether PIF3 serves as a phosphorylation target for phytochrome, are questions which remain to be answered. The independent isolation of an *Arabidopsis* mutant *poc1* with perturbed phytochrome signalling due to an insertion in the promoter of *PIF3* (Halliday et al. 1999) lends credence to the idea that PIF3 is biologically important.

Fankhauser et al. (1999) have identified PKS1 as a substrate phosphorylated by phytochrome. Isolated from a yeast two-hybrid screen, *in vivo* PKS1 is a cytoplasmically-located protein capable of binding phytochrome A. One possibility is that PKS1 acts as a cytoplasmic-anchoring protein that negatively regulates translocation of phytochrome A to the nucleus (see section **2.11**). Phytochrome-mediated phosphorylation of PKS1 could then free the phytochrome A for translocation (Fankhauser et al. 1999, Smith 1999).

2.11 The cellular location of phytochrome

The question of the localization of phytochrome within the cell is associated with those relating to action mechanism and the identification of phosphorylation targets. Sakamoto and Nagatani (1996) used *GUS-PHYB* fusions to demonstrate the presence of a functional nuclear localization sequence in the carboxy-terminus of *Arabidopsis* phytochrome B. Immunoblot analysis confirmed the presence of a substantial fraction of total phytochrome B in isolated WT nuclei. The amount of phytochrome B present in the nuclei decreased following FR light irradiation and in darkness, suggesting the nuclear localization of phytochrome B is a light-dependent process.

Kircher et al. (1999) used a similar approach with Green Fluorescent Protein fusions. Their experiments with rice phytochrome A and tobacco phytochrome B indicate both phytochrome types to be localized in the cytoplasm in darkness. Red light treatment induced nuclear translocation of both phytochrome types (although at different rates) while FR light only induced translocation of phytochrome A and negatively regulated the R-light dependent translocation of phytochrome B.

These reports of differential translocation of the phytochromes are likely to be of great significance in defining action mechanism and reaction partners. Sakamoto and Nagatani (1996) suggest that light-dependent translocation of phytochrome to the nucleus can be regarded as an integral step in the phytochrome signal transduction cascade. Localization of phytochromes within the cell needs to be considered in the context of the evidence of serine/threonine kinase activity (section **2.10**) and identified components of the signal transduction chain (section **2.12**), in order to achieve an integrated understanding of phytochrome action (Frohnmeyer 1999).

2.12 The phytochrome signal transduction network

Following light perception, there must be transduction of the phytochrome-induced signal to downstream components of the associated developmental pathways. Elements of the signal transduction network have been reviewed by Quail et al. (1995), Chamovitz and Deng (1996), Barnes et al. (1997), Mustilli and Bowler (1997), Quail (1998), Whitelam and Devlin (1998),Whitelam et al. (1998), and Deng and Quail (1999). Biochemical and genetic approaches have been combined to provide information about the signalling components.

A biochemical complementation technique involved injection of putative signal transduction molecules into single cells of the phytochrome-deficient *aurea* mutant of tomato (Neuhaus et al. 1993, Bowler et al. 1994). Following the demonstration that injection of phytochrome A results in measurable responses, it was reasoned that active signalling molecules should be capable of eliciting phytochrome-like response in the absence of co-injected phytochrome, and that co-injection of antagonists should then block the phytochrome-like response . These analyses identified an array of potential signalling intermediates; heterotrimeric G-proteins, Ca^{2+}/calmodulin, IP_3 and cGMP are amongst the agents thought to be involved (Barnes et al. 1997). Under certain conditions, these molecules are capable of producing a full photoresponse even in the absence of co-injected phytochrome A. Clearly, these molecules are generic, involved in a diverse range of signalling cascades and not capable of carrying informational specificity in their own right; hence their involvement must occur downstream of specific responses induced by the various phytochrome family members (Bowler et al. 1994, Roux 1994, Barnes et al. 1997).

The identification of signalling-impaired mutants provides an alternative approach to dissecting phytochrome signal transduction. Some recessive signalling-impaired mutants showing an etiolated or partially-etiolated phenotype in the light have been identified in *Arabidopsis*. Mutants *fhy1* and *fhy2* resemble phytochrome A-deficient mutants yet possess normal levels of spectrally active phytochrome A protein and normal levels of *PHYA* mRNA (Whitelam et al. 1993). As such, these mutations may define positively acting downstream components specific to the phytochrome A signal transduction pathway. The *fhy1* mutant lacks only a subset of phytochrome A responses, and so defines a branchpoint in the signal transduction pathway (Barnes et al. 1996b). *Spa1* is thought to represent another phytochrome A branch point (Hoecker et al. 1998). Loci specific to phytochrome B signalling include *red1* (Wagner et al. 1997), *pef2* and *pef3* (Ahmad and Cashmore 1996). *Pef1* (Ahmad and Cashmore 1996) and *psi2* (Genoud et al. 1998) are defective in both phytochrome A and B signalling, indicating that the WT gene products function downstream of convergence of the individual phytochrome signal transduction pathways. It remains possible that each photoreceptor may transduce signals through a minimum of two pathways, one shared and one separate (Deng and Quail 1999). Considerable complexity in the signalling network is indicated by the range of experimental results obtained and components so far identified.

Another informative class of mutants are those showing a constitutive light response; they develop with light-dependent phenotypes even when grown in complete darkness. A large number of such mutants have been characterised (*cop/det//fus* - see Deng and Quail 1999). The general phenotype indicates that the wild-type COP/DET/FUS gene products operate as negative regulators of the overall process of photomorphogenesis. One of the most extensively studied of this group of proteins is COP1, which is nuclear in the dark and becomes cytoplasmic following prolonged exposure to light (von Arnim and Deng1994, Wei and Deng 1996, Torii and Deng 1997).

A smaller number of positive regulators of photomorphogenesis have been identified. As *hy5* mutants develop normally in darkness but fail to respond to illumination by undergoing full de-etiolation (Koornneef et al. 1980), the HY5 transcription factor is classified as a positive regulator (Deng and Quail 1999). HY5 is a bZIP-type DNA-binding protein constitutively localised in the nucleus (Ang et al. 1998, Chattopadhyay et al. 1998). Other putative positive regulators of photomorphogenesis are the two Myb-like transcription factors LHY1 and CCA1

(Schaffer et al. 1998, Wang and Tobin 1998), and CIP7 which was identified through its interaction with COP1 (Yamamoto et al. 1998).

The identification of so many negative regulators and so few positive regulators indicates that photomorphogenesis is the default developmental pathway, and must be repressed in darkness for etiolation to occur (Wei et al. 1994). The COP, DET and FUS proteins are postulated to act as general suppressors of photomorphogenesis. As many primitive plants follow similar developmental pathways whether growing in darkness or light, skotomorphogenesis may be an evolutionary response in higher plants to terrestrial features such as soil and vegetational shade under dense canopies (McNellis and Deng 1995). Photomorphogenesis could thus be considered the original developmental pathway and skotomorphogenesis a specialised developmental program to enhance fitness in darkness and low light conditions (McNellis and Deng 1995).

Deng and Quail (1999) present the following model for light signalling events. Light signals perceived by phytochrome A and B are transduced by distinct pathways to regulate the key developmental repressor COP1 - either directly or by modulation of interactive partners such as COP9, DET1 or COP10 which are of known importance in maintaining COP1 in the nucleus. COP1 is proposed to interact with transcription factors in the dark to prevent gene activation. In light both the inhibitory activity of COP1 and its nuclear abundance is reduced, freeing up transcription factors to induce gene expression via light responsive elements

2.13 Molecular targets of phytochrome signal transduction

Another pertinent aim is to identify those genes which are influenced by phytochrome signal transduction. The measurable input to the phytochrome system is light, and a measurable outcome can be considered to be alteration in mRNA levels or gene product. This may occur either through transcriptional activation or repression of specific genes (Batschauer et al. 1994). Phytochrome-responsive genes may be directly regulated by phytochrome, or indirectly through altered expression of an intermediary gene(s) in a cascade. Similarly, phytochrome may interact directly with DNA/transcriptional machinery of the target genes or via a second messenger cascade. Identification of phytochrome-responsive sequence elements in target promoters, and protein factors binding to these elements, could help in tracing back through the signal transduction network to the initial interaction with the photoreceptor molecule (Quail 1994a).

Identified target genes for phytochrome regulation include chalcone synthase (*CHS*) which is involved in biosynthesis of anthocyanin, a light-protective pigment; and Rubisco (*RBCS*), chlorophyll a/b binding proteins (*CAB*) and ferredoxin-$NADP^+$ oxidoreductase (*FNR*), all of which are required for photosynthesis (Barnes et al. 1997). However, perhaps the best studied target of phytochrome signal transduction is phytochrome A itself. *PHYA* transcript levels in monocots undergo autoregulation; following light exposure there is a marked decline in *PHYA* mRNA levels, attributable to a high intrinsic turnover rate rather than direct photoregulation of transcript stability. Repression of further transcription occurs rapidly without requirement for protein synthesis, and is saturated at low levels of Pfr (Quail 1994a).

By virtue of sequence homology, conserved sequences have been identified in oat rice and maize *PHYA* which may represent control sites for light autoregulation by phytochrome. These are known as Boxes I, II and III. A pair of GT-1 boxes have also been identified (Bruce et al. 1991). 5' deletion studies have delineated 400 bp of oat *PHYA3* necessary for maximal expression in optimal conditions (i.e. darkness) and for Pfr-imposed repression. Within this 400 bp stretch are 3 identified elements. PE1 and PE3 are positive elements which interact in synergistic fashion to allow maximal expression of phytochrome A under low Pfr (i.e. de-repressed) conditions. The third identified element is RE1, which with its presumptive associated repressor-factor 1 RF1 acts as the molecular switch terminating the signalling pathway used by phytochrome to repress the *PHYA3* gene. Mutagenesis in this element results in constitutively maximal expression of *PHYA3* (reviewed in Quail 1994a).

2.14 The future of phytochrome molecular research

A variety of phytochrome gene family members have now been isolated and it is not yet clear whether further phytochrome types remain to be identified. Molecular cloning and mutant studies have allowed the allocation of distinct and overlapping roles to various members of the phytochrome gene family, and have assisted in the interpretation of older biochemical and physiological data. Evidence from biochemical studies and sequencing alignments has converged to indicate that phytochromes most likely function as protein kinases. New understanding of the light-dependent localisation of phytochrome within the cell is combining with the identification of potential interacting factors and phosphorylation targets to generate further insights into the molecular mechanism of action of phytochrome.

Phytochromes are part of a range of photoreceptors used by the plant to integrate signals across the light spectrum, and as such they do not function in isolation. One of the next important tasks for researchers in the field is to more closely define the molecular interaction alluded to by the finding that cryptochrome can act as a substrate for the phosphorylating activity of phytochrome *in vitro* (Ahmad et al. 1998). The ultimate aim will be to answer fully the fundamental questions regarding the mechanistic action of phytochrome, and how phytochrome interfaces with other photoreceptors and mechanisms such as the circadian clock to make its critical contribution to the plant's ability to exist effectively and efficiently in a fixed position in space.

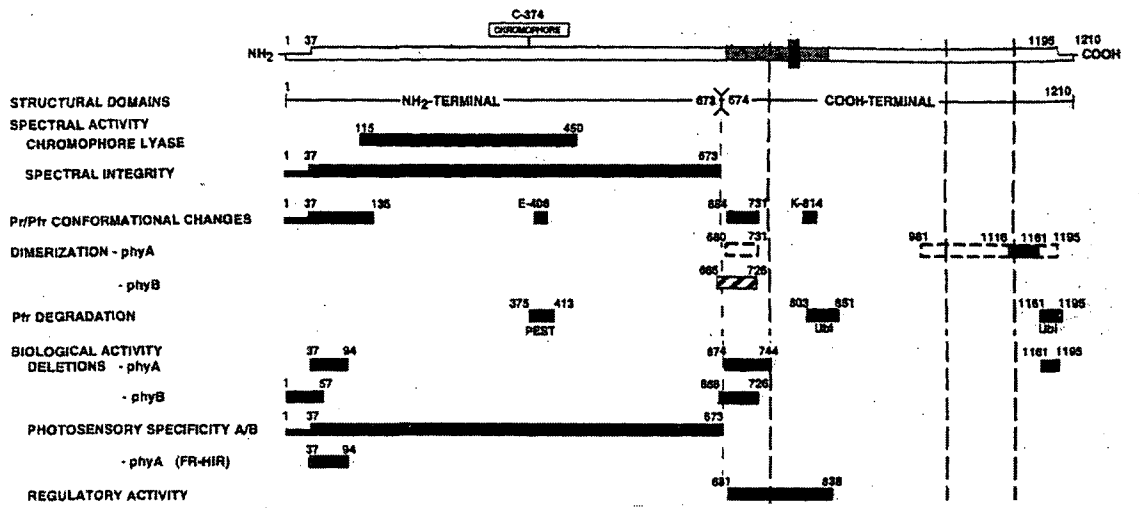

Fig 2.1 A generalised molecular map of phytochrome, for a consensus phytochrome molecule derived from alignment of multiple full length phytochrome amino acid sequences. Co-ordinates for this figure and throughout Chapter 2 relate to the position number of the residues relative to the start position of this consensus phytochrome. The extremities of the figure (positions 1 and 1195) are equivalent to the extremities of *Arabidopsis PHYB*, the longest sequence used in the initial alignment (Mathews et al. 1995). The indented positions at residues 37 and 1195 correspond to the termini of *Arabidopsis PHYA*. Horizontal bars indicate regions of particular activity or properties. The light vertical line indicates the junction of the two major structural domains of phytochrome, while the heavier dashed lines mark the positions where introns occur within the *PHY* structural genes. The stippled box marks a region where multiple missense mutations have been identified, and the black box indicates a region of multiple substitutions of individual residues. This figure has been simplified from Quail (1997).

Chapter 3. General materials and methods

3.1 Introduction

This chapter briefly outlines materials and techniques employed consistently throughout the project, focussing on the genetic material, general growth conditions of the experimental plants and protocols used in the molecular biology laboratory. Specific experimental conditions and method adaptations are presented later in the appropriate chapters. Frequently used abbreviations are listed separately at the beginning of the thesis.

3.2 Plant material

All experimentation utilised garden pea (*Pisum sativum* L.) as the study material, with the approach being to use comparisons between developmental mutants and their progenitor wild type (WT) to investigate aspects of phytochrome A in pea.

The three phytochrome A mutants under investigation in this thesis – *fun1-1*, *fun1-2*, and AF05 – were produced by Dr. James L. Weller in an ethylmethane sulphonate (EMS) mutagenesis program conducted on a cv. Torsdag (HL107) background in Hobart in 1993. Phytochrome mutants were selected utilising red (R) and far-red (FR) light screening of seedlings, and the three phytochrome A mutants identified were initially characterised at the physiological and biochemical levels by Dr. Weller (Weller 1996, Weller et al. 1997a).

The *fun1-1* mutant is characterised by blindness to FR-light, and was first selected on the basis of a dark grown phenotype (pale elongated stem, unexpanded leaves, presence of an apical hook) when grown in the presence of FR light. The phenotype and characterisation are described fully in **Chapter 4**. The allelic *fun1-2* mutant is similar although work carried out with this mutant to date has been less extensive.

The AF05 line carries a dominant mutation which appears to confer exaggerated responsiveness to FR-light. The phenotype and characterisation of the mutant are described in **Chapter 5**.

3.3 Growth of experimental plants

Pea plants grown to maturity were planted singly in 14 cm slim-line pots, while those harvested at the seedling stage were commonly grown at high density in tote boxes (30 x 40 x 12 cm). A small nick (3 - 4 mm^2) was made in the testa above the cotyledon of pea seeds before planting, to promote even imbibition and germination. Seeds were also dusted with a fungicide (Thiram) to guard against infection. Also, pots and toteboxes were sterilised with 70 % ethanol. All peas were grown in a 1:1 (v/v) mixture of vermiculite and 10 mm dolerite chips, topped with 3 - 4 cm of pasteurised pea/sand potting mixture. Pots and boxes were watered immediately prior to and after planting. Following planting, boxes were watered daily until seedling emergence. Watering was then ceased for 3 - 4 days to prevent damage to the emerging apical bud. Once a week plants were treated with the nutrient solution Aquasol ® (Hortico, Sydney, Australia; N:P:K 23:4:18 at a rate of 1 g l^{-1}) plus iron chelate (Kendon Chemicals, Melbourne, Australia) at 0.05 g l^{-1}. The main shoots of pea plants were trained to grow up vertical strings.

3.4 Growth conditions

Generally plants were either grown in a heated glasshouse or in controlled environment cabinets under certain parts of the light spectrum. In cabinets, continuous white light was provided by 40W cool-white fluorescent tubes (Thorn, Australia). Red light was provided by 40W red fluorescent tubes (Tungsram, Hungary). Far-red light was sourced from 20W long-wavelength fluorescent tubes (FL20S-FR74, Toshiba, Tokyo, Japan) filtered through far-red plastic (FRF-700, Westlake Plastics, Lenni, PA, USA). Light fluences and spectra were determined using a LiCor LI-1800 portable spectroradiometer.

In the glasshouse, plants were exposed to an 18 hour photoperiod consisting of natural daylight extended morning and evening by a mixture of fluorescent tubes (L40 W cool white; Osram, Munich, Germany) and incandescent globes (100 W Pearl; Thorn, Melbourne, Australia) delivering approximately 25 μmol m^{-2} s^{-1} at the pot surface. The mean daily temperature range in summer was 17 - 30 °C and in winter was 13 - 21 °C.

When necessary, plants were grown in a dark room with black-painted walls and curtaining to protect against light penetration. The only light used was a dim green safelight, employed occasionally and for limited time periods to carry out manipulations or harvest of the dark-grown plants. This was provided by cool white fluorescent tubes wrapped in blue, yellow and green plastic film (Nakagawa Chemical; cutting sheets 521C, 321C,431C, respectively).

3.5 Extraction of genomic DNA

Genomic DNA was extracted following the procedure described in Ellis (1994). Up to 10 pea leaflets were collected, frozen immediately in liquid nitrogen and ground to a fine powder using a mortar and pestle. The tissue was allowed to warm towards 0 °C, at which stage 10 ml of extraction buffer (3x SSC, 0.1 M EDTA) was added per 5 gram tissue. 100 µl of 20 % SDS was added slowly, while mixing to prevent the SDS from precipitating. The mixture was transferred to a 50 ml Falcon tube and extracted with 15 ml chloroform : isoamyl alcohol (24:1, v/v) by mixing vigorously. The tube was centrifuged (4000 rpm, 10 minutes) at room temperature, and the aqueous layer carefully removed to a fresh tube. This was overlaid with 20 ml of 96 - 100 % ethanol, and the DNA which formed at the interface was spooled onto a glass hook. After airdrying, the DNA was dissolved in 500 µl of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.4) and extracted with 500 µl of phenol equilibrated with TE buffer (pH 8; Sigma). The DNA/phenol mix was centrifuged (10 000 rpm, 4 minutes) and the aqueous layer removed to a clean 1.5 ml microfuge tube. An ethanol overlay was again used to precipitate the DNA which was spooled, rinsed in 70 % ethanol, airdried, resuspended in a minimal volume of water and stored at -20 °C. Water was chosen as the suspension medium rather than TE buffer as buffer components may have interfered with subsequent PCR and sequencing applications. Storage in water necessitated storage at -20 °C rather than 4 °C, so care was taken to minimise the number of freeze/thaw cycles (and potential degradation) that DNA samples were subjected to.

At times, a mini-prep adaptation of this extraction method was used. This began with grinding of a single leaflet in a microfuge tube, and was then essentially as described above, although with suitable reductions in the volume of reagents required. This method is also described by Ellis (1994).

When necessary, DNA concentration was estimated using either a GeneQuant RNA/DNA calculator (Pharmacia) or in a DNA fluorometer (Hoefer Scientific Instruments). An absorbance of 1 unit at 260 nm is the equivalent of a DNA concentration of 50 $\mu g\ ml^{-1}$. DNA was sometimes visualised on 1 - 1.2% agarose horizontal 1x TAE minigels containing ethidium bromide. Either SPP-1 bacteriophage DNA digested with Eco R1 (Bresatec) or DNA Molecular Weight 100 bp Ladder (Low) (Bresatec) were used as size markers.

3.6 DNA blotting

Gels required for blotting were washed for one hour in denaturing solution (1.5 M NaCl, 0.5 M NaOH) followed by one hour in neutralising solution (3 M NaCl, 0.5 M tris HCl pH 6.5) with gentle shaking at room temperature. They were then transferred to Zetaprobe membrane (BioRad) by capillary blotting carried out overnight in 20x SSC (3 M NaCl, 0.3 M Na citrate.2 H_2O), as outlined in Maniatis et al. (1989). Following transfer, filters were rinsed in 2x SSC and DNA immobilised on the membrane by crosslinking in a UV Stratalinker 2400 (Stratagene) at 12 000 μJ x 100 as recommended by the manufacturer.

3.7 Isolation of total RNA

RNAse–free conditions were maintained throughout. Total RNA was isolated from 2.5 g aliquots of powdered apical tissue following the protocol of Michael et al. (1996, modified from Verwoerd et al. 1989). Each sample was snap frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. The frozen powder was transferred to 50 ml Falcon tubes, and 8 ml of TLES buffer (100 mM Tris HCl pH 8, 100 mM LiCl, 10 mM EDTA, 1% SDS) and 8 ml citrate-buffered phenol (pH 4.3, Sigma) added. Samples were then homogenised with a polytron before addition of 8 ml chloroform, then centrifuged at 10 000 rpm for 10 minutes. The supernatant was transferred to a fresh tube, re-extracted with a further 4 ml of phenol and 4 ml chloroform : isoamyl alcohol (24:1, v/v), and again centrifuged at 10 000 rpm for 10 minutes. The aqueous layer was transferred to a baked RNAse-free glass Corex tube and precipitated overnight at 4 °C in an equal volume of 4M LiCl. The sample was then centrifuged at 4 °C for 20 minutes at 10 000 rpm, and the pellet redissolved in 2.5 ml RNAse free water. The RNA was reprecipitated at - 20°C for a minimum of 4 hours in 25 µl of NaAc and 5ml of EtOH, spun 10 minutes at 10 000 rpm at 4 °C, washed in 70% EtOH,

re-spun for a further 10 minutes, air dried, and finally resuspended in a minimal volume of RNAse-free water, generally 200 – 400 µl.

RNA preparations were stored at - 80 °C until ready for use, and quantified from their absorbance at 260 nm using a GBC UV/VIS 916 spectrophotometer. When quantifying RNA, an absorbance of 1 unit at 260 nm indicates a concentration of 40 µg ml^{-1}. Absorbance was also measured at 230 and 280 nm, with a ratio of greater than 1.8 for A_{260}/A_{280} indicating a protein-free preparation, and a ratio of greater than 2.0 for A_{260}/A_{230} indicating the absence of contaminating carbohydrates.

3.8 Northern transfer

Total RNA was electrophoresed on a formaldehyde denaturing gel as described by Fourney et al. (1988). RNA samples were first denatured by heating to 65 °C for 15 minutes in 20 µl of electrophoresis sample buffer with 1 µg ethidium bromide added (stock solution comprises 0.75 ml formamide, 0.15 ml 10x MOPS, 0.24 ml formaldehyde, 0.1 ml H2O, 0.1 ml glycerol and 0.08 ml 10% bromophenol blue). Samples were run on a 1.2% agarose gel containing 0.66 M formaldehyde and 1x MOPS buffer (20 mM MOPS, 5 mM NaAc, 1 mM EDTA), with 1x MOPS as the electrophoresis running buffer.

After electrophoresis gels were soaked in 10X SSC prior to transfer. Gels were blotted onto Genescreen Plus Hybridisation Transfer Membrane (NEN™ Life Science Products) by capillary action overnight, in a solution of 10 x SSC. RNA was fixed to the membrane by crosslinking in a UV Stratalinker 2400 (Stratagene) at 12 000 µJ x 100 as recommended by the manufacturer. The original gel was checked under UV light for completeness of transfer of nucleic acid to the filter, and the position of the ribosomal bands on the cross-linked filter also checked and recorded by photography on a UV light source.

3.9 Preparation of radioactive probes

Suitable gene fragments were amplified in a Perkin Elmer Gene Amp 9600 thermal cycler. The desired fragment was isolated by gel electrophoresis, purified by running through a QiaQuick™ column (according to manufacturer's instructions; Qiagen) and labelled using an MBI Fermentas DecaLabel™ DNA

labelling kit. Approximately 100 ng of template DNA was required. Probes were prepared in accordance with the manufacturer's instructions, and unincorporated radiolabelled dCTP was removed by chromatography on Sephadex™ G-50. Incorporation was estimated using a Geiger counter. All probes were denatured by boiling prior to use in hybridisation reactions.

3.10 Hybridisation and signal detection

DNA blots were prepared for hybridisation by wetting filters in 2x SSC and prehybridising for at least one hour in a solution of 0.5 M sodium phosphate buffer pH 7.2, 1 mM EDTA and 7 % SDS. Northern blots were prepared for hybridisation by dampening in 2x SSC and prehybridising for a minimum of four hours in 5x SSC, 50 % formamide, 1 % SDS and 10% Denhardt's solution. Salmon sperm DNA, used as a blocking agent, was denatured immediately before use by boiling for 10 minutes at 100 °C, and then cooled on ice for a further 10 minutes. 300 µl salmon sperm DNA (10 mg ml^{-1}) was added to each tube of prehybridisation solution.

All hybridisations were carried out overnight in glass tubes, in either a Hybaid Maxi 14 oven or a Hybaid Micro 4 oven. Fresh aliquots of the same solutions used in prehybridisation were used. Hybridisation volume was 5 ml, and temperature was 42 °C for RNA blots and 65 °C for DNA blots. 300 µl freshly denatured salmon sperm DNA (10 mg ml^{-1}) was added to the hybridisation fluid for RNA blots.

Successive washes of increasing stringency (increasing temperature and decreasing salt concentration) were used to remove non-specific radioactivity from the hybridised blot. This process was monitored at all times with a Geiger counter. Typically, 10 quick washes were carried out for each step of the washing procedure with the general protocol being as follows: -
Wash Conditions (1) – 42 °C 2x SSC, 0.1 % SDS
Wash Conditions (2) – 65 °C 0.2x SSC, 0.1 % SDS
For extremely high stringency washes, or to strip a blot for re-probing, 0.1% SDS was used at 65 °C or higher.

Radioactive filters were exposed to autoradiography film (Kodak, Amersham) at -80 °C. Length of exposure time varied according to the amount of radioactivity

bound to the filters, as indicated by a hand-held Geiger counter. X-ray film was developed following standard procedures (Maniatis et al. 1989).

3.11 Polymerase Chain Reactions, sequencing reactions and molecular analysis

All cycling reactions were carried out using a Perkin Elmer Gene Amp 9600 thermal cycler. Unless otherwise stated, the polymerase used was Advantage® cDNA polymerase mix (Clontech), a mixture of KlenTaq1-DNA polymerase and a second DNA polymerase selected to provide 3' to 5' proofreading activity. This enzyme mix is optimised for amplification of long targets and to minimise the errors introduced during the PCR amplification process. It also contains TaqStart™ Antibody to provide an automatic "hot start".

PCR products were visualised on 1.2% agarose horizontal 1x TAE minigels containing ethidium bromide. Either SPP-1 bacteriophage DNA digested with *Eco* R1 (Bresatec) or DNA Molecular Weight 100 bp Ladder (Low) (Bresatec) were used as size markers to check fragments for sizing.

When required for downstream applications such as sequencing, PCR products were purified using Qiagen's QIAquick™ Gel Extraction Kit. A single band of appropriate size produced in PCR reactions was excised from the gel, solubilised at 50 °C in buffer QX1 (Qiagen), and spun through a QIAquick column as outlined in the manufacturer's protocol. The column was washed with buffer PE, the DNA was eluted with 30 µl sterile MilliQ water, and the concentration determined using a DNA fluorometer (Hoefer Scientific Instruments).

PCR derived fragments were sequenced using dye terminator reaction chemistry (Applied Biosystems International) following manufacturer's instructions. The sequencing template generally consisted of 80 - 120 ng of purified PCR product derived from genomic DNA. Details of primers are given in **Chapter 4**. Sequencing was performed on an ABI 370 automated sequencer (Applied Biosystems) by Bronwyn Innes and Sharon Appleyard at the CSIRO Marine Laboratories, Hobart, Tas. or by Dale Levitzke of the Biomolecular Research Facility, Newcastle, NSW. Sequence alignments and examinations were conducted using Sequence Navigator (version 1.0, Applied Biosystems 1989-94), SeqVu (version 1.0.1, Garvan Institute 1992 - 95) and Clustalw (version 1.5).

3.12 Immunoblotting

Total phytochrome was extracted from the apical tips of 7-day old dark-grown pea seedlings. Harvested tips were frozen immediately in liquid nitrogen and stored at - 80 °C until ready for processing. All extractions were carried out under dim green safelight in a cold room. Samples were ground to a fine powder with pestle and mortar, and approximately 100 mg powdered tissue was combined with 150 µl extraction buffer (50 % w/v ethylene glycol, 20 mM $NaHSO_3$, 50 mM ß-mercaptoethanol and 4 mM PMSF) and 10 mg insoluble polyvinylpyrrolidone in the presence of protease inhibitors leupeptin (2 µg ml^{-1}), pepstatin (1 µg ml^{-1}) and aprotinin (2 µg ml^{-1}) and 4 mM iodoacetamide (López-Juez et al. 1992).

Samples were centrifuged at 14 000 rpm for 15 minutes. 100 µl of supernatant was removed to a second tube and combined with 100 µL of electrophoresis loading buffer. Extracts were denatured at 80 °C and subjected to SDS-PAGE in a 6.5% acrylamide gel cast in a Mini-PROTEAN® II apparatus (BioRad). Proteins were electroblotted on to a polyvinylidene difluoride filter (BioRad) in 100 mM tris, 192 mM glycine and 25 % (v/v) methanol. Membranes were blocked with a series of tris buffer/saline – Tween (TBST) washes, as described by López-Juez et al. (1992), and then incubated with the primary antibody in TBST containing 1 % (w/v) skim milk powder. This incubation extended overnight at 4 °C with continuous gentle shaking. The anti-pea phytochrome A monoclonal antibody used was described by Nagatani et al. (1984), and was kindly supplied by Akira Nagatani. After further washing with TBST, membranes were incubated with a 1:5000 dilution of anti-mouse IgG–alkaline phosphatase conjugate (Protoblot, Promega) for one hour at room temperature. Binding of the antibody to the membrane was detected by staining according to the manufacturer's instructions (Promega).

3.13 Chlorophyll assays

2 cm fragments of stem tissue were excised from the apex of seedlings and immersed in 2 ml of dimethylformamide for 24 h at 4 °C. The amount of chlorophyll in the resulting extract was determined spectrophotometrically, using the method of Inskeep and Bloom (1985).

Chapter 4. Molecular characterisation of pea mutants deficient in phytochrome A

4.1 Introduction

The identification and characterisation of mutants specifically deficient or impaired in a single phytochrome apoprotein facilitates the allocation of physiological roles to that phytochrome within the growing plant, and (potentially) elucidation of phytochrome action mechanisms. This is considered perhaps the most direct manner of assigning phytochrome function (Koornneef and Kendrick 1994). Such mutants may be either spontaneously occurring or induced, and are generally selected initially on the basis of altered phenotypic response to certain portions of the light spectrum.

As the first phytochrome identified, and the one most abundant in etiolated tissues, the properties and functions of phytochrome A have been extensively studied. Mutants have made a critical contribution to the pool of knowledge about this phytochrome. Mutants at the *PHYA* locus are blind to FR light and under such light conditions exhibit an etiolated growth form essentially the same as that seen in dark grown plants (Nagatani et al. 1993b, Parks and Quail 1993, Whitelam et al. 1993, van Tuinen et al. 1995, Weller et al. 1997a). Phytochrome A-deficient or impaired mutants have so far been identified in *Arabidopsis* (Nagatani et al. 1993b, Parks and Quail 1993, Whitelam et al. 1993), tomato (van Tuinen et al. 1995) and pea (Weller et al. 1997a). Further information about the role of phytochrome A comes from overexpression studies. Transgenic *Arabidopsis* and tobacco plants which constitutively and ectopically overexpress oat or rice *PHYA* show enhanced sensitivity to FR light and a persistent FR-HIR in light grown seedlings (Boylan and Quail 1991, McCormac et al. 1992, 1993, Whitelam et al. 1992).

Potential phytochrome A-deficient mutants were first identified in *Arabidopsis* by three independent research groups (*fre1,* Nagatani et al. 1993b; *hy8*, Parks and Quail 1993; *fhy2*, Whitelam et al. 1993). In each case, these mutants were initially selected on the basis of a long-hypocotyl phenotype in response to the FR portion of the spectrum. These mutants are specifically deficient in PHYA spectral activity and protein accumulation. *fre1* mutations map close to the known position of the Arabidopsis *PHYA* gene on chromosome 1 (Nagatani et al. 1993b) suggesting that the mutations may lie within the *PHYA* structural gene.

Western blotting indicates the level of PHYA apoprotein in the *fre1-1* mutant is less than 10% of the level occurring in the WT; the level in *fre1-2* is slightly higher, indicating this may be a leaky allele. PHYB accumulates to normal levels, and these mutants retain their responsiveness to R light, indicating the deficiency to be specific to phytochrome A. The simplest and most obvious explanation of the phenotype and biochemical data is that the *fre1* mutants represent a molecular lesion lying within the *PHYA* structural gene itself. Parks and Quail (1993) similarly indicate that mutants *hy8-1* and *hy8-2* lack detectable phytochrome A polypeptide and spectral activity, although a third allele *hy8-3* has normal phytochrome A levels yet lacks activity. Whitelam et al. (1993) report some molecular analysis of *fhy2* mutants and conclude that the three groups of *Arabidopsis* mutants (*fre1*, *hy8*, *fhy2*) are likely to be allelic. Mapping places *fhy2* within the region of the *Arabidopsis* chromosome 1 that carries *PHYA*, the structural gene for phytochrome A. Further, *PHYA* probes generated from WT *Arabidopsis* indicate that the abundance of *PHYA* transcripts in etiolated material is substantially reduced in *fhy2-2* compared with WT. *PHYA* transcripts are undetectable in *fhy2-1*. Restriction fragment mapping identified the presence of a re-arrangement within the *PHYA* gene of the *fhy2-1* irradiation-induced mutants.

Dehesh et al. (1993) demonstrated that the *Arabidopsis PHYA* locus is synonymous with *HY8* by sequencing the *PHYA* gene in lines carrying *hy8* mutant alleles. A single nucleotide change causing an in-frame translational stop codon in the protein coding sequence was identified within the *PHYA* sequence of mutants *hy8-1* and *hy8-2*. This is consistent with the earlier finding that both these mutants lack detectable phytochrome A activity or apoprotein (Parks and Quail 1993). Considered together, these studies (Dehesh et al. 1993, Nagatani et al. 1993b, Parks and Quail 1993, Whitelam et al. 1993) have been fundamental to further work establishing the roles of phytochrome A in regulating growth and development in *Arabidopsis* in response to environmental changes. One major conclusion drawn by each research group relates to the apparent dispensability of phytochrome A for control of photomorphogenesis of *Arabidopsis* plants grown in normal W light (Nagatani et al. 1993b, Parks and Quail 1993, Whitelam et al. 1993).

Phytochrome A-deficient mutants have also been characterised in tomato. Van Tuinen et al. (1995) first reported the identification of mutants *fri1* and *fri2* lacking responsiveness to FR light. Phytochrome A polypeptide cannot be detected immunologically in extracts from these mutants, and the bulk spectrophotometrically detectable pool of light labile phytochrome is also absent

(van Tuinen et al. 1995). Adult plants display retarded growth and a tendency to wilt (van Tuinen et al. 1995). A molecular analysis (Lazarova et al. 1998) has demonstrated these mutations lie within the *PHYA* gene. At the genomic level both *fri* mutants are characterised by an identical base substitution which sees a consensus AG/ replaced by TG/ at the 3' end of the intron between exons 1 and 2. This mutation interferes with correct processing of the pre-mRNA. Some transcripts retain the mutated intron, but cryptic splicing and exon skipping also occur. Analysis suggests that these mutants have less than one percent of the WT phytochrome A level. Despite carrying an identical mutation, the *fri* mutants have distinguishable phenotypes and were selected independently (Lazarova et al. 1998). The identification of a molecular lesion in the *PHYA* genomic sequence in the mutants strongly supports the assumption that the tomato *FRI* locus is the equivalent of the *PHYA* locus.

In pea, the *fun1* mutants (from ***far-red unresponsive***) described by Weller et al. (1997a) are prime candidates for phytochrome A-deficiency caused by a mutation lying within the *PHYA* structural gene. Phenotypically, the *fun1* mutants demonstrate a reduced sensitivity to FR light. Mutant seedlings grown under W or R light are indistinguishable from the WT, but under FR light grow with the pale elongated phenotype characteristic of etiolated plants (Fig. 4.1). In essence, these seedlings can be considered blind to FR light. Interestingly, unlike the situation for either *Arabidopsis* (Nagatani et al. 1993b, Parks and Quail 1993, Whitelam et al. 1993) or tomato (van Tuinen et al. 1995), the phytochrome A-deficient mutants of pea show a marked W light phenotype as mature plants. When grown to maturity under long photoperiods the *fun1* mutant plants display short internodes, thickened stems, delayed flowering and senescence, elongated peduncles and high seed yield – characteristics more common to a WT pea plant grown under non-inductive short days (Weller 1996, Weller et al. 1997a). The phenotypic characterisation of the mature mutant plants indicates that in pea phytochrome A plays a crucial role in the ability of the plant to detect daylength extensions, and hence in control of flowering (Weller et al. 1997a, Weller et al. 1997b). Such a phenotype is absent in the day-neutral tomato and, although a role for phytochrome A in promotion of flowering in *Arabidopsis* under some circumstances has been postulated (Johnson et al. 1994), the distinct phenotypic syndrome described for *fun1-1* plants of pea has not been noted in *Arabidopsis*. Therefore, it seems that full characterisation of the *fun1* mutants of pea has the potential to reveal novel information about (a) roles of phytochrome A in pea (b) control of flowering in pea and (c) interaction between phytochrome-regulated pathways and the floral induction pathway – information which cannot be

deduced simply via the characterisation of phytochrome A-deficient mutants in other species. In pea there is evidence that the pathway for phytochrome action in the mature plant is distinct from that in de-etiolating seedlings (Weller et al. 1997a); the *fun1* mutants provide an opportunity to dissect these potentially independent mechanisms of phytochrome action.

Full utilisation of the *fun1* mutants for physiological studies is dependent on being able to provide a clear and unambiguous explanation of the cause of the phenotype at the molecular level. At the biochemical level, the *fun1-1* mutants are specifically deficient in PHYA apoprotein and lack spectrophotometrically detectable phytochrome (Weller 1996, Weller et al. 1997a). Taken together, the evidence points to the *fun1* mutants being deficient in, and possibly null for, phytochrome A. This thesis reports the characterisation of these mutants at the molecular level, which is essential if these mutants are to be used to their full potential to examine and dissect the roles of phytochromes in garden pea. The approach has been to examine the sequence of the *PHYA* gene in the *fun1* mutants.

The nucleotide sequence of the WT *PHYA* gene of *Pisum* has already been determined from cv. Alaska (Sato 1988; GenBank Accession number M37217). Both cDNA and genomic sequences have been studied, and intron/exon boundaries identified. The pea *PHYA* gene consists of five exons and four short introns, and encodes a protein of 1124 amino acid residues. Southern blot analysis indicates there is a single copy of this gene per haploid genome (Sato 1988), although a pseudogene reminiscent of an incorrect splicing event has also been identified (Sato 1990). Analysis of the 5' leader sequence of the *PHYA* gene indicates the presence of three distinct transcription start sites, generating three transcripts (RNAs 1, 2, and 3) differing in the length of the 5' non-coding sequence (63, 285, and about 465 nucleotides long, respectively) (Sato 1988). Of these, RNA1 is the most prevalent in dark grown plants (when overall levels of *PHYA* are at their maximum).

This chapter assembles the evidence required to demonstrate that *FUN1* is synonymous with the *PHYA* locus in pea.

4.2 Results

4.2.1 There are sequence differences between the WT *PHYA* gene of cv. Alaska and cv. Torsdag

Since the previously determined *PHYA* sequence for pea is from cv. Alaska (Sato 1988), and the *fun1* mutants have been generated on a cv. Torsdag background, it was initially necessary to establish the sequence of *PHYA* in the appropriate WT. The two sequences were found to be near identical, with four neutral changes observed between the published cv. Alaska sequence and the consensus sequence derived for cv. Torsdag. These differences are as follows:

(1) the addition of an A following nucleotide 468 (numbering as for the published Alaska sequence of Sato 1988)
(2) the substitution of an A for a G at nucleotide 1643. This is a translationally silent change, which when read in-frame alters the codon from TCG to TCA. It is predicted that amino acid 151 remains a serine as in the Alaska sequence
(3) the substitution of a G for an A at nucleotide 3020. Again this is a translationally silent difference. The codon becomes GGG instead of GGA , but is still predicted to encode a glycine residue at the position of the 610th amino acid
(4) the addition of an A following nucleotide 5217

These changes are highlighted in Fig. 4.2. As changes (1) and (4) are outside the amino acid coding region, and changes (2) and (3) are silent alterations that do not effect the amino acid sequence, these differences between the Torsdag and Alaska sequences are likely to be of little (if any) practical importance. This means that intron/exon boundaries and transcription start sites for cv. Torsdag *PHYA* can be deduced from the cv. Alaska sequence.

4.2.2 The *PHYA* gene of *fun1-1* plants contains a premature translational stop codon

As Fig. 4.3 indicates, direct comparison between the Torsdag WT sequence and the sequence of the *PHYA* gene from the *fun1-1* mutant revealed the presence of a point mutation at nucleotide 2783. This result was obtained for three independent sequencing events. When read in the frame of the cDNA sequence, the single base substitution converts the codon encoding the 531st amino acid (tryptophan, TGG) to a premature translational stop signal (TGA). This is predicted to result in

the production of a truncated and presumably inactive phytochrome A of 530 amino acids in length – only 47% of the full-length protein. The carboxy terminal of the phytochrome is essentially absent, leading to a loss of signal transduction activity (Quail 1997; see section **2.8**). Although the truncated protein retains a domain identified as important to chromophore lyase activity, determinants conferring A/B photosensory specificity and spectral integrity are predicted to be absent. This result is consistent with phenotypic and biochemical indications of the absence of any detectable, active full-length phytochrome A polypeptide in this mutant (Weller 1996, Weller et al. 1997a).

4.2.3 The *PHYA* gene of *fun1-2* plants also contains a premature translational stop codon

A similar situation was found for the mutant *fun1-2*. This mutant was found to contain a point mutation at nucleotide 1365 of the *PHYA* gene, which when read in frame converts the codon encoding the 59th amino acid (glutamine, CAA) to a premature translational stop signal (TAA) (Fig. 4.4). This result was obtained in three independent sequencing experiments. This single base substitution is predicted to lead to the production of a truncated and presumably inactive phytochrome A of 58 amino acids in length – only 5 % of the full-length protein. These first 58 amino acids are believed to contain the region specifying photosensory specificity for the phytochrome A FR-HIR (Quail 1997, Fig. 2.1), but other critical regions – including those conferring spectral integrity, chromophore lyase activity, ability to undergo the Pr/Pfr conformational change, dimerization determinants and signal transduction activity – are all predicted to be absent from the *fun1-2* phytochrome A. Again, this result is consistent with prior phenotypic and biochemical indications of the absence of any detectable, active full-length phytochrome A polypeptide in this mutant (Weller 1996).

4.2.4 The *fun1-2* mutant phenotype and the molecular lesion in the *fun1-2 PHYA* gene co-segregate

An analysis was conducted to determine whether the *fun1-2* phenotype and the molecular lesion identified in the *fun1-2 PHYA* gene co-segregate. The point mutation identified in the *PHYA* gene of the *fun1-2* mutant results in the loss of a restriction site for the enzyme BsaB I which is present in the WT (cv. Torsdag) sequence. A 776 bp PCR fragment containing this stretch of sequence would therefore be expected to generate a distinct restriction pattern following digestion with BsaB I, in accordance with whether the fragment has been amplified from

WT or mutant genomic DNA. In theory, DNA from WT plants will be cut into two fragments of 540 and 236 bp. DNA from mutant plants will remain uncut (776 bp). Heterozygous plants with a WT phenotype would be expected to generate bands of all three sizes (776, 540 and 236 bp).

DNA was extracted from 36 members of F_4 families segregating WT and *fun1-2* plants, as well as from control plants of each parental line, and subjected to PCR and restriction digest. Of the 36 segregating plants grown, 11 were classified as mutant on the basis of their FR light phenotype (chi square for a 3:1 ratio = 0.59, 0.5 >P>0.3). An analysis of the restriction pattern generated showed perfect correlation between mutant phenotype (elongated stem under FR light) and molecular lesion, with all 11 phenotypically-mutant plants displaying a single, uncut 776 bp fragment indicative of loss of the BsaB I restriction site (see Fig. 4.5). This provides further proof that the *fun1-2* mutation lies within the *PHYA* structural gene in pea. As *fun1-2* and *fun1-1* have previously been shown to be allelic (Weller 1996, Weller et al. 1997a), this situation naturally extends to include *fun1-1*.

Under the conditions of the co-segregation analysis, heterozygous and homozygous WT plants were indistinguishable due to incomplete digestion of the genomic DNA, resulting in the presence of bands of all three possible sizes (776, 540 and 236 bp) in the lanes containing DNA from all plants with a WT phenotype. However, there was no opportunity for confusion between WT and mutant as in no case did an individual identified as having a mutant phenotype generate any bands other than the uncut 776 bp amplified DNA fragment. Similarly, no individual classified as WT on the basis of phenotype failed to generate multiple bands in the restriction digest.

4.2.5 Immunodetectable phytochrome A is absent from *fun1-2* seedlings

Immunochemical studies have previously shown that there is no detectable phytochrome A apoprotein present in extracts from dark-grown *fun1-1* plants (Weller et al. 1997a). The present study has extended this to include crude extracts from dark-grown *fun1-2* plants. Anti-pea phytochrome A antibody mAP5 (Nagatani et al. 1984) failed to detect the presence of PHYA in extracts of 7 day-old etiolated *fun1-2* seedlings (Fig. 4.6), whereas in WT seedlings an intense band was detected at approximately 121kDa. Some smaller, fainter bands were also detected by the antibody; these probably represent products of partial proteolysis (Lopez-Juez et al. 1992). Phytochrome B was present in *fun1-2*

mutants at levels equivalent to those seen in the WT (data not shown) as determined using antibody mBP1 (formerly mAP11; Konomi et al. 1987). These results are consistent with those obtained previously for *fun1-1* (Weller et al. 1997a) and are in accordance with what would be expected in a mutant specifically deficient in active full-length phytochrome A.

4.2.6 *PHYA* transcript levels are reduced in *fun1-1* and *fun1-2* seedlings

The transcript size and abundance of *PHYA* in the WT and *fun1* mutants were investigated using a species-specific, gene-specific probe. No size differences were noted between the WT and mutant transcripts. The probe used (see section **4.4.6**) hybridised to a full-length *PHYA* of approximately 4.2 kb. As anticipated, after taking gel loading into account, the abundance of *PHYA* was greater in RNA extracted from dark-grown plants than in light-grown seedlings. Expression of the *PHYA* gene appeared to be greater in the WT than in the mutant seedlings in both light- and dark-grown plants (Fig. 4.7). The lower abundance of transcript in the mutants suggests that the respective single base substitutions causing premature stop codons in *fun1-1* and *fun1-2 PHYA* are sufficient to also cause reduced stability of the mRNA transcripts. The level of *PHYA* transcript is lowest in the extracts from *fun1-1* plants, indicating this transcript to be even less stable than that of the *fun1-2* plants (Fig. 4.7).

It was not possible to test the level of other phytochromes present in the pea RNA preparations at this stage. The only other member of the phytochrome gene family confirmed to be present in pea is *PHYB* (N. Beauchamp, pers. comm.) and a probe and method of sufficient sensitivity to detect this phytochrome by Northern blotting are yet to be developed.

4.3 Discussion

4.3.1 The *FUN1* locus is the *PHYA* structural gene

The physiological and phenotypic evidence (Weller 1996, Weller et al. 1997a) indicate that the *FUN1* locus is in all probability the *PHYA* structural gene. The etiolated phenotype of *fun1* mutant seedlings grown under FR light, the lack of spectrophotometrically detectable bulk light-labile phytochrome in the *fun1-1* mutant, and the absence of immunochemically detectable PHYA in extracts from *fun1-1* seedlings all point towards a defect in the *PHYA* structural gene in both of the allelic *fun1* mutants. The definitive demonstration of this fact, however, is

reliant on the presentation of sequencing data. It is shown here that point mutations are present within the *PHYA* gene of the *fun1-1* and *fun1-2* plants. Co-segregation between the *fun1-2* mutant phenotype and the molecular lesion identified in the *PHYA* gene of *fun1-2* plants has also been demonstrated, indicating the phenotype and the molecular lesion to be inseparable. This is as would be anticipated for a situation where the molecular lesion is the **cause** of the observed phenotype. These pieces of evidence, combined with the earlier physiological and phenotypic studies (Weller 1996, Weller et al. 1997a), establish firmly that the *FUN1* locus of pea is the *PHYA* gene.

It could be argued that final, formal proof would require complementation of the *fun1* mutant phenotype by introduction and expression of the WT *PHYA* gene in mutant plants. While such a process is routinely carried out in *Arabidopsis* and is also feasible in tomato, in pea this remains technically challenging, and is unwarranted in this situation given the strength of the existing evidence that the *FUN1* and *PHYA* loci are synonymous in pea. Indeed, the present study already provides more proof than presented for *HY8* in *Arabidopsis* (Dehesh et al. 1993) and *FRI* in tomato (Lazarova et al. 1998) by virtue of the demonstration of co-segregation between mutant phenotype and molecular lesion.

4.3.2 The truncated phytochrome A proteins of *fun1-1* and *fun1-2*

The predicted phytochrome A proteins of the *fun1* mutants lack so much of the information contained in the full-length amino acid sequence that the failure to detect any phytochrome A-like activity in these mutants (Weller 1996, Weller et al. 1997a) is not surprising. The *fun1-1* and *fun1-2* mutants have very similar phenotypes under glasshouse conditions, and in all likelihood both these mutants represent phytochrome A nulls. In particular, the severely truncated phytochrome A of *fun1-2* mutants (58 amino acids, 5 % of full-length) can be concluded to retain no residual activity. This allele would therefore be the obvious choice for use in further studies predicated on the assumption that no active phytochrome A is present. This is supported by the results of the Western blotting experiments, which detect WT levels of PHYB in the *fun1-2* mutant but fail to detect any PHYA apoprotein. In itself, the failure of mAP5 to bind to epitopes in the crude protein extract of *fun1-2* does not prove conclusively that there is no PHYA present. This is because the precise location within the protein of the epitope to which the antibody reacts has not been determined. To prove immunologically that PHYA is completely absent would require the use of a range of anti-phytochrome A antibodies which react with different determinants. However, in

the current context the results of the Western blots can be considered in conjunction with the predictions of the sequencing results and the physiological and phenotypic results, to strengthen the conclusion that there is no active phytochrome A present in the *fun1-2* mutant. The identification of the presumptive premature stop codon allows the prediction of the length of the truncated protein, and – through reference to previous mapping of determinants of phytochrome activity (reviewed in **Chapter 2**, and in Quail 1997) – the identification of those regions critical to activity which are absent in the *fun1* proteins.

4.3.3 Expression of *PHYA* in *fun1-1* and *fun1-2*

The absence of WT protein activity can sometimes be explained by a reduction in gene expression, leading to a corresponding reduction in the amount of protein produced. Such an explanation need not be invoked in the case of the *fun1* mutants, as the presence of premature translational stop codons causing the production of truncated proteins would in itself be expected to result in the observed lack of phytochrome A activity. In other words, the absence of activity can be explained by the cessation of protein translation, rather than by irregularities at the transcriptional or post-transcriptional modification stages. However, the observation that *PHYA* mRNA levels are reduced in the *fun1* mutants relative to the WT indicates that there may be some additional contribution to the phenotype at the transcriptional level. Alternatively, it may indicate regulation of transcription by translation, such that transcript stability is somehow reduced by the presence of the premature stop codon.

This situation is not unprecedented. Dehesh et al. (1993) examined the expression of *PHYA* in *Arabidopsis hy8* mutants, and found *PHYA* mRNA levels to be significantly lower in *hy8-1* and marginally lower in *hy8-2* compared to the WT. No similar occurrence was noted in tomato; however, the circumstances are different as the *fri* mutation occurs at an intron/exon boundary, resulting in disruption of normal splicing and the production of transcripts of variable size in the mutants (Lazarova et al. 1998). The decrease in *PHYA* expression seen in the pea and *Arabidopsis* mutants is not intuitively expected in a situation where the identified molecular lesion causes the introduction of a presumptive translation stop codon. Such a single nucleotide substitution would not necessarily be expected to cause any change to stability or abundance of mRNA. The simplest explanation for the absence of detectable phytochrome A protein or activity in the

fun1 and *hy8* mutants is that the premature stop codons result in production of a truncated and inactive protein.

However, reduced *PHYA* gene expression may make some synergistic contribution to the mutant phenotype. As noted by Dehesh et al. (1993) in the case of the *Arabidopsis hy8* mutants, the reduction in mRNA levels is, alone, insufficient to account for the apparent complete absence of functional photoreceptor molecule. Somers et al. (1991) have noted similar discrepancies for the *hy3* mutants of *Arabidopsis* where severe reductions in phytochrome B level are accompanied only by a 2 – 3 fold decrease in *PHYB* mRNA levels. This is suggested to reflect reduced transcript stability somehow induced by premature termination of translation (Somers et al. 1991), a phenomenon which has also been noted in some other plant gene systems (Daar and Maquat 1988, Vancanneyt et al. 1990). The exact mechanism of this potential regulation of transcription by translation is uncertain but fascinating. Similarly, the relevance of the downregulation of *PHYA* expression in mutants that are in any case incapable of producing active, full-length phytochrome A is unclear at this stage. It can not be stated conclusively whether the decreased expression of *PHYA* in the *fun1* mutants is a simple consequence of the premature cessation of protein translation, or a contributing factor to the *fun1* mutant phenotype. Indepth study of this situation may generate important insights into the relationship between transcription and translation. In the pea mutants, another interesting question yet to be addressed is whether the *PHYA* mRNA levels in known heterozygotes fall midway between the maximal expression typical of homozygous WTs, and the reduced levels in the *fun1* (*phya phya*) mutant plants. It also remains unclear why the *PHYA* transcript levels are lower in *fun1-1* than they are in *fun1-2* (Fig. 4.7).

4.3.4 Differences between the *PHYA* gene sequence of cvs. Torsdag and Alaska

As noted in section **4.2.1**, the differences between the *PHYA* sequences of cvs. Torsdag and Alaska are inconsequential in terms of activity, as they do not cause any alteration to the predicted protein sequence. It is, however, interesting that there is such divergence between the two cultivars. Again, a precedent for this was found in *Arabidopsis* when Dehesh et al. (1993) reported 4 differences between the previously published Columbia *PHYA* sequence (Sharrock and Quail 1989) and the newly defined RLD ecotype *PHYA* sequence. Two of these changes are translationally silent, causing no change in the phytochrome A polypeptide sequence between the ecotypes. One change was determined to be a result of a sequencing error in the previously published sequence, and the final

discrepancy may represent a genuine ecotype difference between the Columbia cDNA and the RLD genomic sequence. In contrast, Lazarova et al. (1998) reported no differences between the *PHYA* sequences of UC82B and MoneyMaker in tomato.

In pea, it is likely that the sequence variations observed are a consequence of the independent development of the two cultivars in question. While cv. Alaska consists of over 100 strains and was initially bred in the USA for canning, cv. Torsdag originated in Sweden and was bred for production of combining and split peas (I. Murfet, pers. comm.). The two cultivars can be considered unrelated and display distinct flowering behaviours. Another pea gene has recently been sequenced from both cultivars, and five differences were noted between the two sequences (L. Schultz, pers. comm.). No attempt was made during the present study to verify the accuracy of the published sequence for the *PHYA* gene of Alaska (Sato 1988), both because it was difficult to establish a suitable seed source for this generic cultivar, and because the published sequence for cv. Alaska *PHYA* was defined rigorously by sequencing six independent cDNA clones in addition to a genomic clone (Sato 1988). In any case, the differences noted have no anticipated functional effect. However, the Torsdag sequences reported here have been verified independently numerous times, and have never shown variation from the sequence now presented (Fig. 4.2). Thus this can be considered the definitive sequence for the WT *PHYA* gene of pea cv. Torsdag.

4.3.5 The chemical cause of the nonsense mutations in the *fun1* plants

The *fun1* mutants were selected from a batch of cv. Torsdag seed treated with the alkylating agent ethylmethane sulfonate (EMS) (Weller 1996, Weller et al. 1997a). This mutagen causes specific mispairing of bases by addition of an ethyl group. It most frequently targets purines and is primarily found to act by creation of an O-6-alkylguanine (G), leading to direct mispairing with thymine (T) rather than its normal partner cytosine (C). This results in GC-to-AT transitions in the next round of replication (Griffiths et al. 1993, Mathews and van Holde 1990, Weaver and Hedrick 1992).

This study has identified a G-to-A transition in the *PHYA* gene of *fun1-1*, and a C-to-T transition in *fun1-2*. Hence both molecular lesions identified are consistent with the type which would be expected to be generated in an EMS mutagenesis program. This is in contrast with the finding of Lazarova et al. (1998); although the *fri* mutants of tomato were selected subsequent to an EMS treatment of a seed batch, the molecular lesion detected involves an A-to-T transversion. Since the

mutation identified in tomato is inconsistent with the described mode of action of EMS as a mutagen, and two *fri* mutants were found to carry the same lesion, it remains a possibility that the tomato mutants actually occurred spontaneously and were uncovered co-incidentally in the mutagenised seed batch. Such a scenario does not apply to the *fun1* mutants of pea, since they each carry a distinct mutation consistent with the EMS mode of action.

4.3.6 Conclusions

Taken together, the results indicate that the *FUN1* locus of pea corresponds to the *PHYA* gene. The dual identification of nonsense mutations within the *fun1-1* and *fun1-2 PHYA* structural genes provides a molecular explanation for the absence of detectable phytochrome A polypeptide or activity in these mutants. The demonstration of co-segregation between the *fun1-2* mutant phenotype and the identified molecular lesion (as tracked by a PCR-based RFLP) is further firm evidence that the lesion in the *PHYA* gene is the cause of the *fun1* phenotype. The failure to detect PHYA apoprotein in extracts of *fun1-2* by Western blot (Fig. 4.6) is consistent with the failure to detect PHYA in *fun1-1* (Weller et al. 1997a) and with the conclusion that introduction of a premature translational stop codon will result in production of truncated and inactive phytochrome A protein.

The results of the molecular characterisation of the *fun1* mutants are consistent with the results of previous phenotypic, biochemical and spectrophotometric studies. These mutants can now be used confidently in conjunction with presumed phytochrome B null mutants of pea (Beauchamp et al. unpublished) in rigorous analyses to further investigate the physiological and developmental roles of the phytochrome family in pea. This potential will be discussed in detail in **Chapter 7.**

4.4 Materials and methods

4.4.1 Plant material

The *fun1-1* and *fun1-2* mutants were selected following an ethylmethane sulfonate (EMS) mutagenesis program conducted on a cv. Torsdag (HL107) background in Hobart in 1993. Seeds were treated in a 1% (v/v) EMS solution for 6 h at room temperature, and mutant selection was as described in Weller et al. (1997a), using a combination of red and far-red light screening of seedlings. The two allelic

mutants identified were initially characterised at the physiological and biochemical levels (Weller 1996, Weller et al. 1997a).

4.4.2 Amplification of the *PHYA* gene using Polymerase Chain Reaction (PCR)

The size of the genomic *PHYA* gene in pea (5694 nt, including introns; see GenBank Accession number M37217) rendered it unsuited to reliable amplification as a single fragment. Following many attempts at optimisation, the decision was made to amplify the gene from genomic pea DNA in three overlapping fragments:

(1) the first 2450 bp of the gene, including the 5' untranslated region as published by Sato (1988)
(2) the final 3060 bp of the gene, including the 3' untranslated region as published by Sato (1988)
(3) the region from nt 2350 to nt 3350, to ensure correct sequencing of the middle portion of the gene

Standard PCR conditions were used in reactions, with a typical 50 µl reaction comprising 40 µl sterile MilliQ water, 5.µl Klentaq buffer (Clontech), 1 µl 10mM dNTPs, 1 µl of each primer (forward and reverse) at 20 µM, 1 µl genomic DNA template at a concentration of approximately 50 ng/µl and 1 µl Klentaq enzyme (Clontech). The primer pairs and cycling conditions used to amplify each portion of the phytochrome A gene are as follows:

(1) phyA start (forward primer) 5'ATA CGA TGA CAT GGC ATC ACT ATC ATT CAG 3'
and middle reverse (reverse primer) 5' TAC AAT ACC TAA GGG TGC ATC TCG CAT CAA 3'
Cycling parameters:
1min at 94 °C
then 35 cycles of 15 sec at 94°C
3 min at 68 °C
then 3 min extension at 68 °C
followed by a 15°C soak.

(2) middle forward (forward primer) 5' CTG TTG TGT GAT ATG TTG ATG CGA GAT GCA 3'

and phyA end (reverse primer) 5' GCT GTG ATG GAT TGG AAG AAA GAC ACT ATT 3'

Cycling parameters:
1min at 94 °C
then 35 cycles of 15 sec at 94 °C
3 min at 68 °C
then 3 min extension at 68 °C
followed by a 15°C soak.

(3) gap upper (forward primer) 5' AGT TTC TGG CTC AAG TGT TT 3'
and gap lower (reverse primer) 5' ATC CCC ATG TGT TTT TAT CTC 3'
Cycling parameters:
1 min at94 °C
then 10 cycles of 15 sec at 94 °C
30 sec at 57 °C
60 sec at 68 °C
then 20 cycles of 15 sec at94 °C
30 sec at 52 °C
60 sec at 68 °C
followed by a 15 °C soak.

4.4.3 Sequencing reactions and molecular analysis

Sequence information was derived from the PCR products amplified from genomic pea DNA. Reactions were prepared as described in section **3.11**. Sequencing primers were designed from the available Alaska sequence (Sato 1988, GenBank Accession no. M37217) at intervals of approximately 350 bp in order to generate overlapping sequenced fragments encompassing all exons and introns of the *PHYA* gene, extending from upstream of the translational start site to the extreme 3' end of the published sequence. The complete list of sequencing primers used is given in Table 4.1.

Sequences obtained were compared with the cv. Alaska *PHYA* gene (Sato 1988). Where sequence differences were observed between the Alaska sequence and the Torsdag WT or mutants, the region containing the differences was re-sequenced several times, including at least once from an independent DNA preparation (i.e. harvested from a second group of plants). This was done to confirm that a real change in sequence was being observed, rather than simply a sequencing error or

PCR artefact. Use of an enzyme mix with proof reading ability provided further insurance against errors.

4.4.4 Co-segregation analysis

36 plants, representing four F_4 families segregating for WT and *fun1-2* mutant phenotype, were grown for 7 days under FR light and assigned as either WT or mutant on the basis of stem length. Following transfer to W light and subsequent greening, DNA was prepared from each individual as well as from five control plants of each parental line. A 776 base pair fragment of the *PHYA* gene was amplified from each of these DNA preparations, using the following primers:
Coseg Upper (21-mer) 5' GCA GGT GCA CGG TAT GGT ATG 3'
Coseg Lower (23-mer) 5'GGC GCA GTG AAA ACA GTC CTT AT 3'
The cycling conditions were:
1 min at 94 °C
then 35 cycles of 15 sec at 94 °C
30 sec at 55 °C
1 min at 68 °C
followed by a 15 °C soak.

Following purification and quantification, 0.2 μg PCR product was combined with 10 units of BsaB I (NEB, Queensland) in a total volume of 20 μl. Digestion was for three hours at 60° C. The recognition site for the restriction enzyme BsaB I is 5' GATNN (cut) NNATC 3', present in the Torsdag 776 bp fragment at position 540 (... GATGG cut AGATC...). Hence digestion of the Torsdag PCR product generates two fragments of 540 and 236 bp. Contrastingly, the C-to-T substitution in the sequence of the *fun1-2* PCR product results in loss of the BsaB I restriction site (... GATGG AGATT...) and the 776 bp fragment remains uncut.

Restriction digest products were electrophoresed, blotted and detected using uncut 776 bp PCR fragment from the WT (cv. Torsdag) *PHYA* gene as the probe.

4.4.5 Western blotting

Phytochrome A apoprotein was detected using mAP5, an anti-pea phytochrome A monoclonal antibody (Nagatani et al. 1984). Phytochrome B was detected with mBP1(formerly mAP11; Konomi et al. 1987) which was raised against a pea type 2 phytochrome purified from green plants (Abe et al. 1985). Both antibodies were gifts from Akira Nagatani.

4.4.6 Northern blotting

Northern blotting was carried out according to the protocols outlined in **Chapter 3**. *PHYA* was detected with a 995 bp fragment of WT pea *PHYA* amplified from genomic DNA using the following primers:
AaprF (26-mer) 5' ACT GCA GGG TGA AGA AGA GAA GAA TG 3'
AaprR (25-mer) 5' GGA CCT GCT GAA GCC TAA GAC TAT C 3'
The cycling conditions were:
1 minute at 94 °C
then 35 cycles of 15 sec at 94 °C
30 sec at 56 °C
1 min at 68 °C
followed by a 15 °C soak.

This fragment detected a major *PHYA* transcript of approximately 4.2 kb. Although Sato (1988) has reported the presence of three *PHYA* RNA transcripts of varying length in pea, in that study the transcripts were resolved through detailed mapping of 5' termini, rather than through Northern blotting. The probe used in the current experiments did not resolve the three distinct transcripts.

The gene fragment used as a probe does not contain any introns, and spans the linker region between the phytochrome A amino- and carboxy-termini, incorporating the "core" region containing a high density of identified missense mutations, and the region identified as conferring the regulatory activity of phytochromes (Quail 1997). This is also a region highly conserved amongst members of the phytochrome family (Quail 1997). The possibility of cross-reaction with phytochromes other than phytochrome A is low, however. Hybridisation conditions were stringent, as for a gene specific probe. The only other member of the phytochrome family which has been conclusively identified in pea is a *PHYB* (Beauchamp et al., unpublished) and this gene is expressed at such low levels that it cannot be detected in Northern blots probed with fragments of the cloned *PHYB* gene. Attempts to amplify other members of the phytochrome family from pea using a PCR-based method (adapted from Mathews et al. 1995) have so far failed to produce any further phytochrome-related sequences (N. Beauchamp, pers. comm.).

Table 4.1 Sequencing primers and start position relative to the Alaska sequence. All sequences are given in the 5' to 3' direction.

seqA - GTC ATG TTT TCT CAG TAA C (from nucleotide 314)
seqB - CCA CAC CTT CAC ATG GG (nt 620)
phyA upper - CTT GAA CTT GAA CTT TCA CAA CAG CCA AGT (nt 905)
seqC - GAG GCC TAG CCA ATC GTG (nt 1201)
seqD - GAG ATG CTG ACT ATG GTG (nt 1502)
seqE - GCT AAA GCA ATT ACA AG (nt 1802)
seqF - GAT TTG ACT CTG TGC GG (nt 2124)
forward - CTG GCT CAA GTG TTT GCC (nt 2354)
seqG - GGC TTT CCT TGA AGT TG (nt 2851)
seqH - ACT TGG CAC TGC AGG GT (nt 3230)
seqI - CGC GAG GAG GTG ATG GA (nt 3636)
seqJ - GCG AAT CGT GAA CAC TA (nt 4061)
seqK - GTT ATA GCA GCC TCC TT (nt 4446)
seqL - AGC TCA TAA GTT GAA AG (nt 4829)
seqM - TAC TCA CAG CAC TGA CA (nt 5279)
last - TTG TCC TTA AGG TCA AAG (nt 5451)

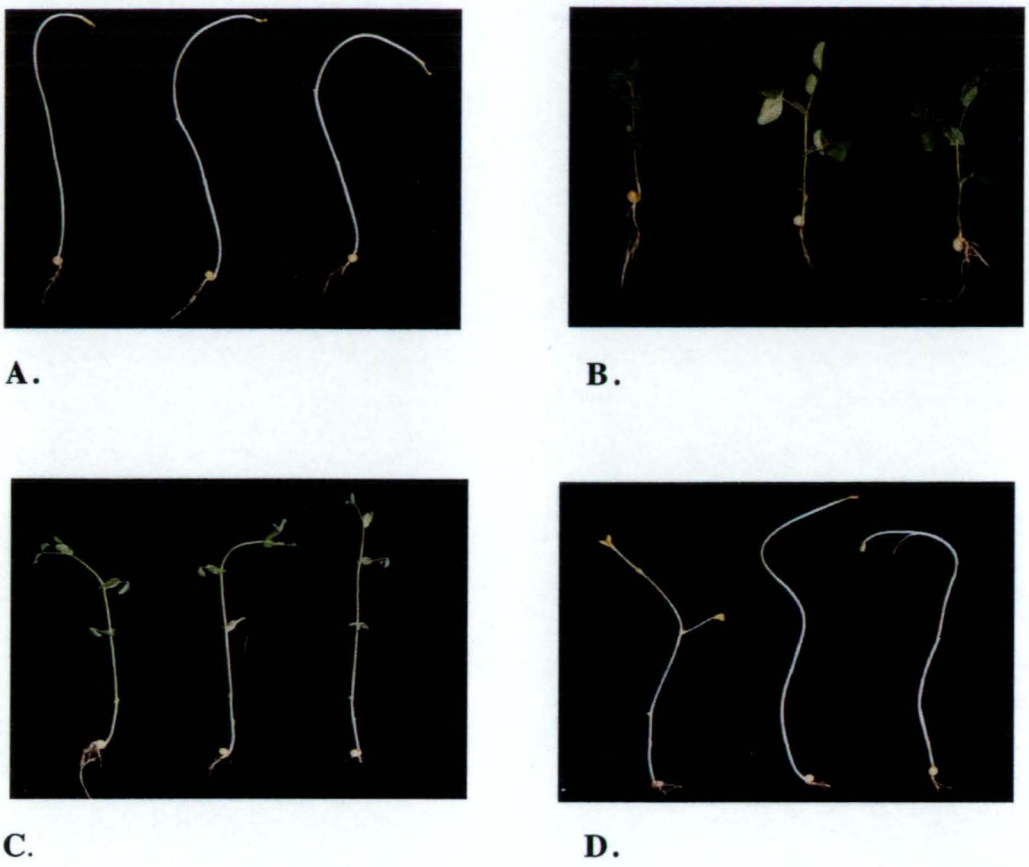

Fig. 4.1 Phenotypes of WT and *fun1* mutant plants grown under a range of light environments. All seedlings are 10 days old. From left to right, genotypes are WT, *fun1-1* and *fun1-2*.

A. Seedlings grown in complete darkness.

B. Seedlings grown in continuous W light (150 μmol $m^{-2}sec^{-1}$)

C. Seedlings grown in continuous R light (20 μmol $m^{-2}sec^{-1}$).

D. Seedlings grown in continuous FR light (8 μmol $m^{-2}sec^{-1}$). The *fun1-1* and *fun1-2* mutants display the classic etiolated phenotype of a WT plant grown in complete darkness (compare with **Fig 4.1A**).

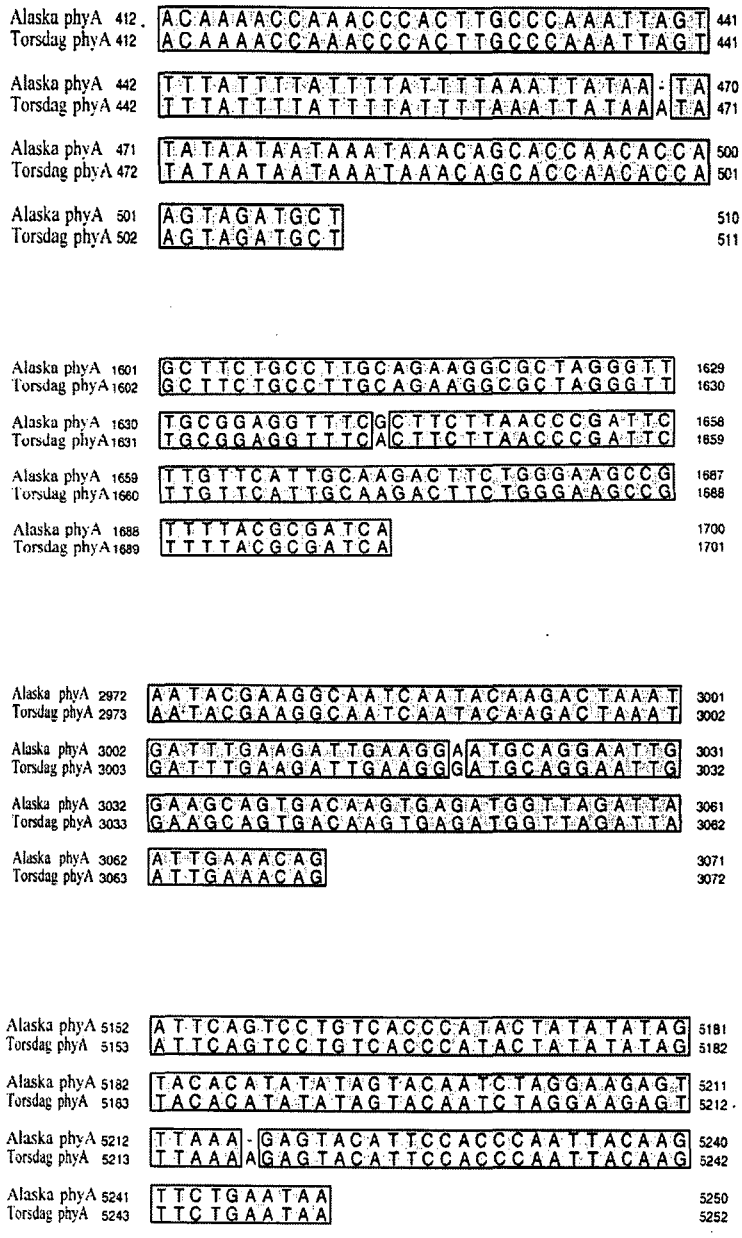

Fig 4.2 Four fragments of the *PHYA* gene from cv. Torsdag, aligned against the previously published sequence from cv. Alaska (Sato 1988). Numbering of nucleotides is based on the numbering of Sato, as entered in GenBank (Accession no. M37217). The full sequence of 5690 nucleotides can be viewed in GenBank. Here the four (translationally silent) differences between the Alaska and Torsdag *PHYA* sequences are indicated.

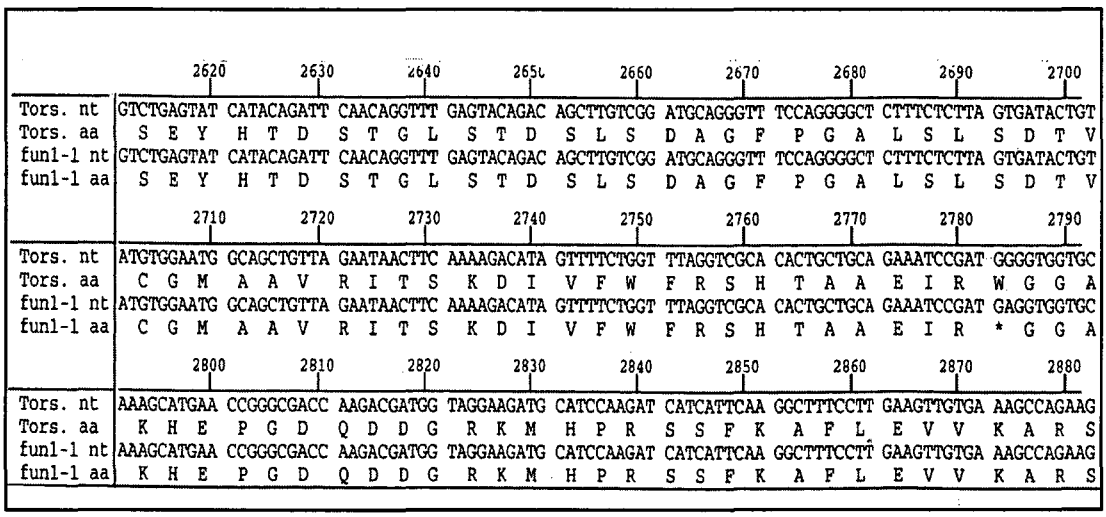

Fig 4.3 A portion of the nucleotide sequence of the *PHYA* gene from the *fun1-1* mutant, aligned against the cv. Torsdag sequence. A single mismatch between the sequences is shown. This base substitution results in the early introduction of an in-frame translation stop codon (*) and, presumably, leads to production of a truncated and inactive phytochrome A molecule.

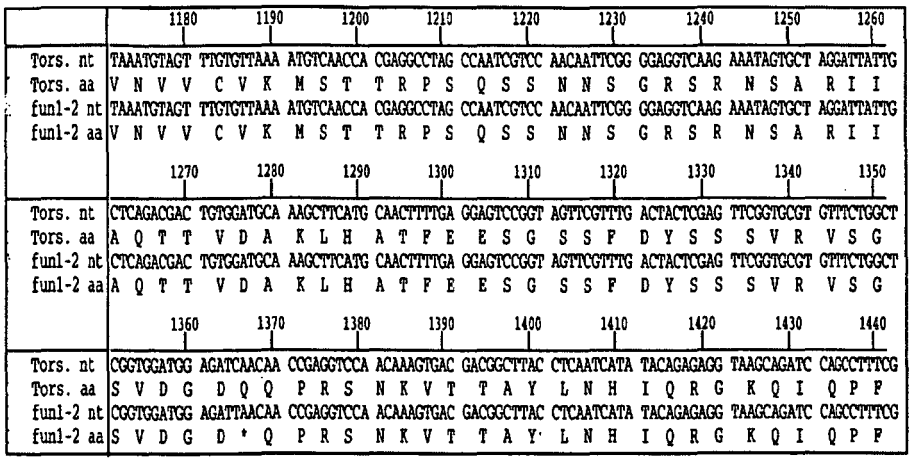

Fig 4.4 A portion of the nucleotide sequence of the *PHYA* gene from the *fun1-2* mutant, aligned against the cv. Torsdag sequence. A single mismatch between the sequences is shown. This base substitution results in the early introduction of an in-frame translation stop codon (*) and, presumably, leads to production of a truncated and inactive phytochrome A molecule.

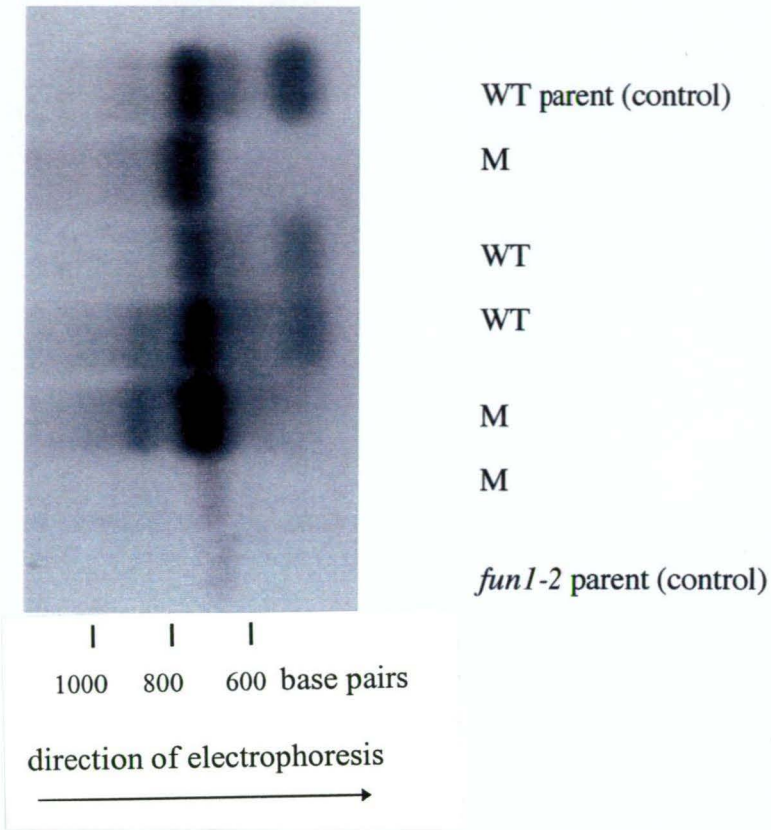

Fig. 4.5 A representative Southern blot showing the restriction patterns for 5 members of the segregating WT/*fun1-2* population used to demonstrate co-segregation of the mutant phenotype and the molecular lesion identified within the *PHYA* gene of mutant plants. DNA from one plant of each parental line is also shown. Lanes containing a single band of 776 bp result from loss of the BsaB I restriction site. Multiple bands indicate the presence of the restriction site found in the WT *PHYA* fragment. Phenotypes of the source plants for the DNA, as determined in the glasshouse, are designated WT or mutant (M).

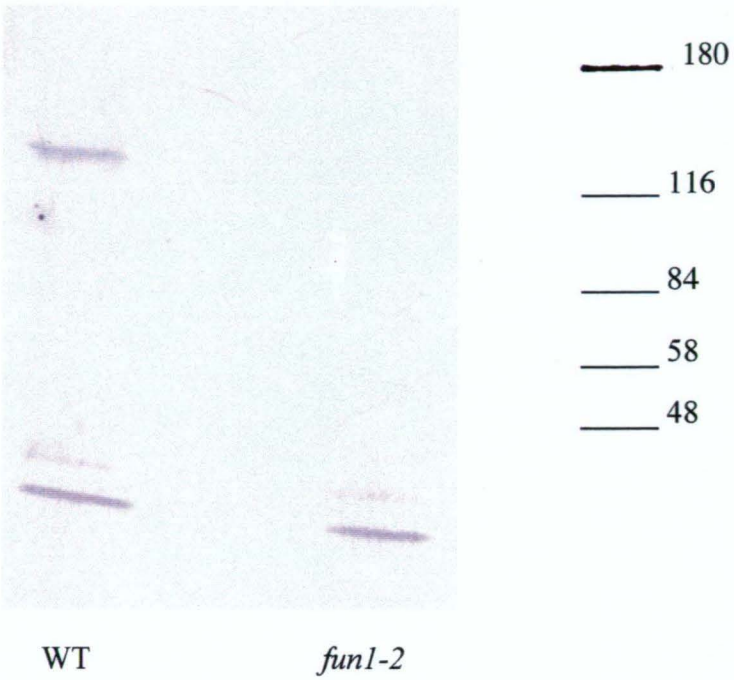

Fig. 4.6 Immunoblot detection of PHYA apoprotein in crude protein extracts from 7-d-old etiolated wild-type cv. Torsdag (WT) and *fun1-2* seedlings. Each lane contains extract equivalent to 2.5 mg fresh weight. The positions and molecular masses (kD) of prestained markers (Sigma) are indicated by black horizontal lines. PHYA is detected as a single band of approximately 121 kD by the monoclonal antibody mAP5 (Nagatani et al.1984). Identical results were obtained in four separate experiments; PHYA apoprotein is absent from *fun1-2* seedlings.

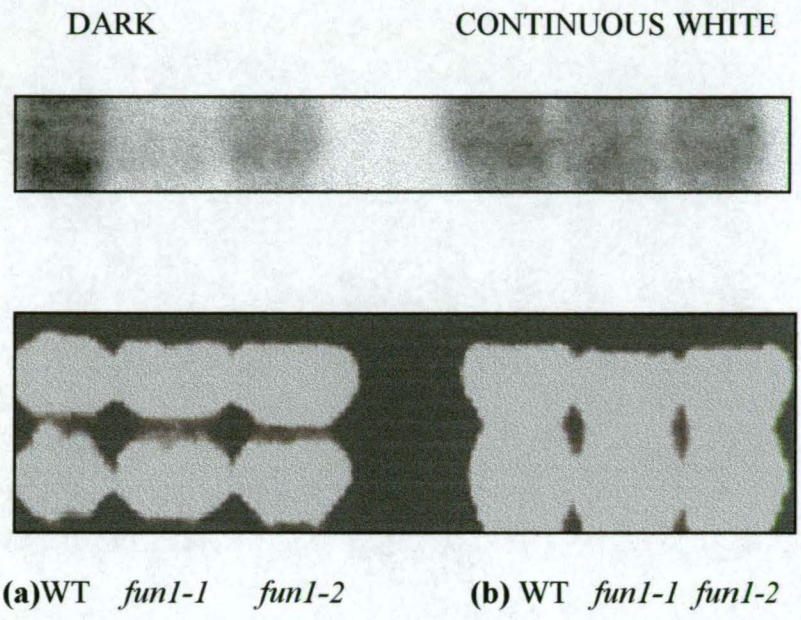

Fig 4.7 *PHYA* mRNA levels in total RNA extracts from WT, *fun1-1* and *fun1-2* seedlings, grown for ten days in either complete darkness or continuous white light. The major *PHYA* transcript was detected as a 4.2 kb band (top panel). Each lane contains approximately 10 µg of total RNA. The bottom panel of the figure shows equivalent loading of dark grown samples **(a)** onto the denaturing gel, and of continuous white light grown samples **(b)**. Relatively more RNA appears to be present on the blot for white grown samples. Similar results were obtained in four independent experiments.

Chapter 5. A molecular investigation of AF05, a mutant with exaggerated light responses

5.1 Introduction

In **Chapter 4** pea mutants deficient in phytochrome A were investigated. The concerted study of the residual light responses, and the light responses altered or absent in such mutants, has facilitated definition of the roles of phytochrome A in environmentally-moderated growth and development. However, a deeper understanding of this phytochrome's role could be gained by a complementary study of mutants with an opposite phenotype.

This issue has been approached by the creation and analysis of transgenic plants that overexpress *PHYA* genes. When phytochrome gene constructs are expressed at sufficient levels, this generally results in a light-dependent phenotype characterised by heightened sensitivity to light. This can include reduced hypocotyl/stem elongation, decreased apical dominance and enhanced pigmentation when transgenic plants are grown under natural light conditions (e.g. Boylan and Quail 1989, 1991, Kay et al. 1989, Cherry et al. 1991, Wagner et al. 1991, Boylan et al. 1994). For example, transgenic tomato and *Arabidopsis* seedlings overexpressing an oat *PHYA* cDNA under the control of the cauliflower mosaic virus 35S promoter are hypersensitive to FR with respect to inhibition of hypocotyl elongation and cotyledon expansion (Boylan and Quail 1989, Whitelam et al. 1992). Oat phytochrome A overexpression in transgenic tobacco confers a dwarf phenotype (Cherry et al. 1991, Jordan et al. 1995, Halliday et al. 1997); the expression of heterologous phytochromes A, B or C in transgenic tobacco plants has been shown to alter both vegetative development and flowering time (Halliday et al. 1997). Transgenic *Arabidopsis* seedlings that overexpress phytochrome A are early flowering under short-day conditions, showing a reduced response to photoperiod (Bagnall et al. 1995).

Although a range of clever experimental techniques has been employed, these studies are not necessarily as informative as characterisation of a phytochrome A-overexpressing mutant might be. Transgenic studies suffer from the difficulties of achieving expression only in those tissues in which endogenous phytochrome would occur, uncertainty about the level of overexpression which will be achieved in any given experiment, and at times unpredictable interactions between the introduced and endogenous phytochromes.

Unfortunately, only a small number of mutants have been described with light-hypersensitive aspects to their phenotype, and none can be specifically attributed to phytochrome A overexpression. Mutants such as *lip1* in pea (Frances et al. 1992) and the *cop/det/fus* class of *Arabidopsis* mutants (reviewed in Deng and Quail 1999) have a de-etiolated phenotype even in darkness. Since the phenotype of these mutants is not light-dependent, they are not examples of mutants with exaggerated response to light, but instead represent a class of mutations in master regulator switches that act in the WT to repress photomorphogenesis. Other mutants do show light-dependent exaggerated photoresponses but no firm link has been established between them and the phytochrome genes. For example, the tomato *hp-1* mutant is hypersensitive to R and FR but does not map near to known phytochrome genes of tomato (van Tuinen et al. 1996) so is unlikely to affect photoreceptor activity directly. Instead, the mutation is presumed to act in some way to alter/enhance phytochrome signal transduction. Preliminary reports of the isolation of *Arabidopsis* mutants specifically hypersensitive to FR light (designated *eid* for **e**mpflindlicher **i**n **d**unkelroten Licht = hypersensitive in FR light; Dieterle et al., European Symposium on Photomorphogenesis 1999) have not yet been followed by publication.

The AF05 mutant of pea, however, appears to be a strong candidate for an overexpression phenotype caused by some mutation in the *PHYA* gene. Weller (1996) identified the AFO5 mutant in a FR-light screen where it showed enhanced de-etiolation compared with the WT. Fig 5.1 shows the AF05 phenotype compared with the WT under a range of environmental conditions. Indistinguishable from WT in darkness, under R, FR and W light AFO5 seedlings show shortened internodes and enhanced leaf development. The distinctive phenotype continues for AFO5 mutant plants grown to maturity in the glasshouse. Mature AF05 plants are shorter than WT and, under non-inductive short days, flower and senesce early relative to the WT (Weller 1996). The phenotype of these mutants is, in effect, the converse of the *fun1* mutants (described in Weller et al. 1997a and reviewed in **Chapter 4**).

AF05 is a dominant mutation, and as described by Weller (1996) represents a novel class of induced mutant. It is similar in both phenotype (enhanced light responses) and mode of inheritance (dominant) to previously described transgenic phytochrome A-overproducing lines. Dissection of R and FR light responses in this mutant suggests a specific enhancement of phytochrome A responses but not enhancement of phytochrome B activity (Weller 1996). AFO5 maps to within at least five map units of *FUN1 (PHYA)* and no recombinants (i.e. WT phenotypes)

were observed among a population of 61 F2 plants generated from a cross between AFO5 and *fun1-1*. Instead, a 3:1 segregation of AFO5:*fun1* types was observed, suggesting that the two mutants are closely linked and quite possibly allelic (Weller 1996).

The evidence suggests that the AFO5 phenotype may result from a direct lesion within the *PHYA* gene, possibly in the promoter region. Therefore, this chapter reports an investigation of the genomic sequence of *PHYA* from AFO5 mutant plants, and of the levels of *PHYA* mRNA and PHYA apoprotein in AF05 plants.

5.2 Results

5.2.1 No mutation was detected within the coding region of *PHYA* in AFO5

The coding region of the *PHYA* gene from AFO5 was sequenced as described for the *fun1* mutants (**Chapter 4**). No sequence variation was noted between the DNA sequence of WT (cv. Tors) *PHYA* and that of AFO5. The coding region was sequenced in its entirety three times from independent preparations of DNA. AFO5 plants used as the DNA source were grown to maturity in the glasshouse alongside WT and *fun1* plants, in order to confirm that they showed the described mutant phenotype. The method used to sequence the *PHYA* gene had already been proven robust by the identification of single point mutations in *fun1-1* and *fun1-2* (**Chapter 4**).

5.2.2 Extended sequencing of the 5' and 3' leader sequences also failed to reveal any differences between the WT and AFO5 sequence

Particular attention was paid to sequencing the flanking DNA both upstream and downstream of the protein coding region. A total of 1189 bp upstream of the translation start site and 635 bp downstream of the stop codon of the main open reading frame (ORF) were sequenced. Again, no variation was detected between the WT and AFO5 sequences.

5.2.3 Levels of *PHYA* mRNA and PHYA apoprotein in AFO5

Weller (1996) measured the levels of spectrophotometrically detectable phytochrome in WT and AFO5 etiolated plants, and followed the changes in these levels over the course of six hours de-etiolation under R light. The phytochrome level in WT fell more quickly than that of AFO5, with a half-life of only 84

minutes compared with 154 minutes for AFO5. The present study investigated *PHYA* mRNA levels and PHYA apoprotein levels in the AF05 mutant.

In dark-grown plants, the expression of the *PHYA* gene was similar in both WT and AFO5 plants (Fig. 5.2). AFO5 plants grown under continuous W light conditions also showed levels of *PHYA* transcript equivalent to those in WT plants (Fig. 5.2). Levels were then assessed in de-etiolating plants transferred from darkness to R light, over a 4 hour time course. At time zero, the amount of transcript detected in each genotype was similar (Fig. 5.3). After 2 hours, and again after 4 hours, the transcript level fell slightly relative to the initial level detected in dark-grown plants, although the decrease was not marked. No clear differences in transcript level were observed between AFO5 and WT plants at any time point. *PHYA* signal intensity appeared slightly greater in AF05 than WT both 2 and 4 hours after transfer (Fig. 5.3), possibly indicating enhanced persistence of the transcript in AF05 after R light exposure. However, the differences in signal intensity are small and may simply be attributable to variation in the amount of RNA loaded on to the gel (see tubulin loading control, Fig. 5.3).

The levels of PHYA apoprotein in WT and AFO5 plants were assessed over a similar time course (Fig. 5.4). At time zero, qualitatively similar amounts of PHYA apoprotein were detected by antibody mAP5 (Nagatani et al. 1984) in crude extracts of both AFO5 and WT plants. In extracts taken both 2 and 4 hours after transfer from darkness, the level of PHYA apoprotein remaining in AFO5 plants was similar to that detected in the WT plants. Under these conditions of R light illumination, the native phytochrome protein is expected to decay through form-specific degradation after conversion to the P_{fr} form. After 2 and 4 hours exposure to R light the amount of apoprotein detected decreased slightly relative to the initial (dark-grown) levels for both genotypes, although the decrease was not strong, and significant apoprotein signal was still detected 4 hours after transfer from darkness.

5.3 Discussion

5.3.1 The *PHYA* structural gene of AFO5

The AFO5 phenotype is consistent with exaggerated phytochrome A responses. The simplest explanation of the described AFO5 mutant phenotype would be the presence of a molecular lesion within the *PHYA* gene, either within the coding

region itself or within the 5' or 3' untranslated regions, such that regulation of transcription, translation or response is somehow affected.

The presence of a mutation within the coding region which could be demonstrated to co-segregate with the AF05 mutant phenotype would simultaneously provide a molecular explanation of the phenotype and conclude the debate about whether AFO5 and *FUN1* are allelic. For example, domain mapping in *Arabidopsis* has already identified a "gain of function" effect which can be caused by deletion of a serine-rich region of *PHYA* between positions 42 – 51 (Jordan et al. 1995a), or site-directed substitution of all these serines by alanine residues (Stockhaus et al. 1992). These changes to WT sequence lead to enhanced phytochrome A activity. Mutation within this region was considered a possible cause of the AFO5 phenotype, but no such mutation was identified, despite repeated checking of the genomic DNA sequence of *PHYA* from the mutant line.

5.3.2 5' and 3' flanking DNA of AFO5 *PHYA*

The next most obvious thing to test for was the presence of a mutation within the promoter. Detailed analysis of the WT *PHYA* promoter has been carried out in oat (Bruce and Quail 1990), rice (Kay et al. 1989a, b), maize (Christensen and Quail 1989), *Arabidopsis* (Sharrock and Quail 1989, Dehesh et al. 1994), pea (Sato 1988) and tomato (Lazarova et al. 1998).

The 5' flanking regions of dicot *PHYA*s are characterised by the presence of short upstream open reading frames (uORFs). Such uORFs have been identified in zucchini (Sharrock et al. 1986), pea (Sato 1988), *Arabidopsis* (Sharrock and Quail 1989), potato (Heyer and Gatz 1992), tobacco (Adam et al. 1995) and tomato (Lazarova et al. 1998); and it has been speculated that the encoded short peptides might participate in translational regulation of the transcripts in which they occur (Dehesh et al. 1994, Sharrock and Quail 1989, Heyer and Gatz 1992). Short ORFS have previously been shown to exert regulatory effects on translation of downstream coding sequences in other situations (Mueller and Hinnebush 1986). These uORFS are considered unusual in plant genes, as are upstream introns (Futterer and Hohn 1996). The presence of these features in the phytochrome 5' sequences is therefore associated with the potential for exerting translational control (Futterer and Hohn 1996, Gallie 1996). However, the AFO5 phenotype is unlikely to be related to potential products of the uORFS since the regions of sequence encompassing these show no variation between WT and AFO5.

It also seems unlikely that the AFO5 phenotype is caused by a mutation further upstream in the pea *PHYA* promoter than has been sequenced in this study. AFO5 sequence invariant from the WT has been determined for 1189 bp upstream of the translation start site of the major ORF that encodes the pea PHYA apoprotein. This 1189 bp fragment of the pea *PHYA* 5' flanking DNA is sufficient to confer full phytochrome A-like light-repression of transcription on *GUS*-fusions expressed in transgenic petunia (Komeda et al. 1991).

For oat and rice *PHYA*s the minimal promoters have been defined by 5' deletion analyses as being of lengths substantially shorter than 1189 bp (Bruce and Quail 1990, Ni et al. 1996). Analysis of the oat *PHYA3* promoter resulted in identification of a negative element RE1 apparently necessary for Pfr mediated down-regulation of *PHYA* expression. Loss of the RE1 element by directed mutagenesis leads to constitutively maximal *PHYA* expression (Bruce et al. 1991). A positively acting element PE3 was also identified (Bruce et al. 1991). Dehesh et al. (1994) highlight a sequence motif ATGGG which is perfectly conserved in the maize, rice, oat, *Arabidopsis* and pea *PHYA* promoters; this motif is at the core of the oat *PHYA3* RE1 element CATGGGCGCGG. Also, the *Arabidopsis* sequence immediately upstream of the conserved ATGGG motif shows strong similarity to PE3 of oat *PHYA3*, and both segments combine to comprise a sequence element Box1 previously identified as being conserved amongst the monocot *PHYA* promoters (Christensen and Quail 1989; Fig. 5.5)

An exciting hypothesis was that the AFO5 phenotype results from mutation within the RE1-like element, preventing normal (WT) Pfr-dependent down-regulation of *PHYA* expression. However, no such mutation was detected. An alternative explanation, which is not discounted by the sequencing data presented in this chapter, is that the AFO5 mutation lies within the gene coding for a putative *trans*-acting repressor which normally binds to the RE1-like element following R light illumination. This hypothesis is not easily reconciled with the mapping and allelism testing indicating the closeness of AF05 and *FUN1/PHYA* (Weller 1996). If the AFO5 mutation lies within a repressor of *PHYA* expression, the gene encoding the repressor must be closely linked to the *FUN1* locus.

The 5' flanking DNA sequenced for AFO5 *PHYA* includes the three identified transcription start sites (Sato 1988), the associated TATA boxes (Sato 1988), and the elements homologous to the PE3 and RE1 elements of oat (Bruce and Quail 1991, Dehesh et al. 1994) and the Box 1 motif conserved in maize, rice and oat *PHYA* promoters (Christensen and Quail 1989). It also incorporates the identified short uORFs (Sato 1988; Fig. 5.5) and AT-rich sequences conserved between

Arabidopsis and pea but of unknown functional significance (Dehesh et al. 1994). The consensus *Arabidopsis* sequence presented by Dehesh et al. (1994) includes an additional 690 nucleotides upstream of the 1189 bp sequenced in AFO5, but no additional elements affecting transcription or activity have been identified in this extra section of flanking DNA. Hence there is no precedent set in any of these well studied systems which would lead to the belief that *cis*-elements essential to pea *PHYA* activity are located further upstream than the point sequenced to in the present study. The only well-characterised exception to this is tobacco, in which maximal *PHYA* expression requires the presence of major *cis*-elements located between –1760 and –1229 upstream of the first translated codon, and possible additional elements both up and downstream. However, these are not novel elements but putative equivalents (T-PE1, T-PE3 and T-GT2) of the *cis*-regulatory elements in oat *PHYA3* (Bruce et al. 1991). For whatever reason, in tobacco these elements are located at a greater distance from the start codon than they are in pea, *Arabidopsis*, oat, rice and maize (see Fig 5.5).

The question arises of how far downstream it is necessary to sequence in search of a mutation causing the AFO5 phenotype. At this point, sequence has been determined 635 bp downstream of the stop signal ending the major ORF. This encompasses the polyadenylation sites and signals (Sato 1988). No variation has been detected between the AFO5 and WT sequences, and there are no descriptions of altered phytochrome activity or response mediated by changes in this region of sequence in other species.

5.3.3 Possible causes of the AFO5 phenotype

It seems fairly clear that the AFO5 mutation does not lie within the *PHYA* gene. Several alternative hypotheses may be invoked to explain the AFO5 phenotype. It is possible that the phenotype results from a mutation in a tightly-linked modifier gene of *PHYA*. The *trans*-acting factor for RE1 described in **5.3.2** is a hypothetical example of such a gene. A similar explanation has been advanced for the action of the novel dominant *Arabidopsis* mutant *fry1*, which has a phenotype similar to that of phytochrome A-deficient mutants. Sequencing has failed to reveal any lesions within the *PHYA* gene of mutant plants, despite the fact that the *fry1* locus maps very close to *PHYA*. Instead, the mutation is hypothesised to lie within a closely-linked gene that modifies phytochrome response (Fry and Deng 1999). Since large numbers of loci are known to be involved in phytochrome signal transduction pathways, some of them specific to

phytochrome A signal transduction (see **Chapter 2.12**), the existence of closely-linked modifier genes is reasonably likely.

An alternative suggestion is that the AFO5 phenotype results from mutation in a *PHYA* enhancer element located at a greater distance from the *PHYA* coding region than accounted for by the genomic AF05 sequence determined here. Comparison with *PHYA* promoters from species other than pea (see section **5.3.2**) does not support this hypothesis, as known regulatory elements have been covered in the region sequenced. To sequence further up or downstream in AFO5 *PHYA* would require use of a different method to the simple PCR of genomic DNA employed in this study.

A third possibility is that the AF05 phenotype is the result of a *PHYA* epi-mutant. Changes in the methylation status of a gene may greatly influence expression, with methylation increasingly being recognised as an important mechanism of gene control (for review see Finnegan et al. 1998). An example of this phenomenon is the gain of function epi-mutant *fwa* of *Arabidopsis* which causes late flowering (Soppe et al. 1999). *FWA* was cloned by a map-based approach, and expression studies indicated that the *FWA* gene was expressed in the *fwa* mutant but not in WT plants. However, the sequence of the *FWA* gene is identical for WT and *fwa-1* mutant alleles. Promoter analysis of *FWA* revealed two repeats, of 30 and 200 bp. Bisulfite sequencing of these regions showed that all cytosines falling within these repeats are methylated in the WT allele but not in the mutant alleles. The differential level of methylation is restricted to these repeat regions. It is assumed, therefore, that the *FWA* gene is not expressed in WT plants due to the hypermethylation of the repeats occurring in the promoter. In the mutants, hypomethylation in this same region results in expression of *FWA* accompanied by the late flowering phenotype. The *FWA* gene is therefore considered to encode for a repressor of flowering (Soppe et al. 1999).

Another well documented example is the range of epi-mutations in the *SUPERMAN* gene of *Arabidopsis* (Jacobsen and Meyerowitz 1997, Rohde et al. 1999). A group of independent mutants named *clark kent* were identified, so named for a phenotype similar to but weaker than previously characterised *sup* mutants. Analysis indicated *clk* and *sup* mutants to be very closely linked, and a genomic clone containing the *SUP* coding region was able to complement the *clk* phenotype in transgenic plants. The evidence pointed to *clk* and *sup* being allelic; however, sequencing of the *SUP* coding region and promoter region in the *clk* alleles revealed no nucleic acid sequence differences from the WT. This led the authors to consider the possibility that the *clk* alleles represent an alternative

epigenetic state of the *SUP* gene, and subsequent analysis revealed extensive methylation in the *SUP* gene of the *clk* mutants (Jacobsen and Meyerowitz 1997).

The possibility of differential methylation of the *PHYA* gene in WT and AFO5 could be tested reasonably easily by restriction digests. Pairs of isochizomers such as Msp I and Hpa II show differential sensitivities to methylation so are expected to generate different restriction patterns depending on the levels of methylation present. If significant differences were indicated by the restriction enzyme assay, the actual distribution of methylated residues in the gene could be determined by bisulfite sequencing (Feil et al. 1994).

However, if a change in methylation status in the *PHYA* gene were the cause of the AFO5 phenotype, significant differences in *PHYA* expression might be anticipated between the WT and AFO5 plants. This is not borne out by Northern blots which indicate the level of *PHYA* mRNA to be largely similar in etiolated plants (Fig. 5.2) and in de-etiolating plants (Fig. 5.3) when de-etiolation was followed over the course of four hours.

5.3.4 Levels of *PHYA* mRNA and PHYA apoprotein in AFO5

Exaggerated phytochrome A-like responses, as seen in AFO5 mutant plants, could potentially be caused by enhanced response to normal levels of phytochrome A, or by the presence of increased amounts of phytochrome. In turn, increased amounts of phytochrome might be present due to enhanced gene transcription, altered stability of transcripts, or altered turnover of the protein product. These factors may well interact in complex fashion.

Spectrophotometric measurements indicate that the depletion of total phytochrome during de-etiolation occurs more slowly in AFO5 than in the WT (Weller 1996). This raises the possibility that phytochrome A apoprotein and transcript levels might be persistent or elevated in AFO5 plants. However, the current study does not provide firm evidence for differential regulation of *PHYA* transcript levels or PHYA apoprotein levels in de-etiolating AFO5 seedlings when compared to the WT.

One challenge is to assess the degree of difference in transcript or protein levels which would need to be seen in order to provide an explanation for the observed phenotype. There is some evidence that only small differences in phytochrome level are required to exert dramatic effects on phenotype. Wester et al. (1994) found in

Arabidopsis that introducing a 2-fold excess of phytochrome B into a WT background is sufficient to induce a phenotype similar to a heterologous overexpressor line with phytochrome B levels 15 times higher than normal. Cherry et al. (1992) showed the phenotypic consequences of oat phytochrome A overexpression in tobacco to be maximal at levels only 2 to 3 times greater than endogenous phytochrome levels. Based on this evidence, only small increases in endogenous phytochrome A level might be required to produce the AFO5 phenotype.

From the current study, it cannot be stated conclusively whether or not the level of *PHYA* transcript or PHYA apoprotein is marginally increased in AFO5 relative to the WT. The Northern and Western blotting results presented do not provide sufficient resolution to answer this question. However, more recent results (Kerckhoffs et al. unpub.) indicate no difference between *PHYA* transcript levels in WT and mutant. In the absence of detectable mutations in the coding region of AFO5 *PHYA*, it is clear that more detailed characterisation of phytochrome gene expression in AFO5 is required to further address the question of the cause of the "overexpressor" phenotype these plants display.

5.3.5 Conclusions

No mutations were detected in the coding region or flanking DNA of AFO5 *PHYA*. A high degree of confidence in the method used to seek mutations is warranted given the success in identifying mutations within the *PHYA* coding sequence of both the *fun1-1* and *fun1-2* mutants (**Chapter 4**). Consideration of the promoters of other *PHYA* genes and important elements within these would tend to indicate that a sufficient amount of flanking DNA has been sequenced to uncover the presence of a mutation in AFO5 *PHYA* if present. An alternative explanation for the AFO5 phenotype, which does not invoke direct mutation of the PHYA gene, must therefore be sought. Hypotheses worthy of further consideration include the suggestions that AFO5 is a *PHYA* epi-mutant with altered levels or patterns of cytosine methylation; that the AFO5 phenotype results from mutation in a closely-linked modifier gene; or that the AFO5 phenotype results from mutation in an enhancer element located further up or downstream than sequenced to date. Since Northern and Western blotting fail to reveal any strong differences in expression of the *PHYA* gene or apoprotein levels between WT and AFO5 mutant plants, the strongest indications are that the mutation in

AFO5 is affecting response to phytochrome A rather than production or turnover of phytochrome A itself; or that the mutation lies in a different phytochrome.

Given the failure of the present study to demonstrate at the molecular level that the phenotypes of AF05 and *fun1* plants result from mutations in the same gene, it will be necessary to return to larger populations of plants and use genetic methods to further test their linkage and allelism status. The discovery of even a single recombinant between AF05 and *fun1* would throw significant doubt on the hypothesis that they are allelic and help to explain the failure to find a mutation in the *PHYA* gene of AFO5.

As stated previously, the sequencing results presented in this chapter provide negative evidence for the hypothesis that the AFO5 mutation affects the *PHYA* gene directly. Although a range of alternative explanations for the phenotype has been advanced, at the current time there is no strong evidence favouring any of them. This is disappointing, given the possible importance of the AF05 phenotype to understanding flowering in pea (Weller 1996). Phenotypic analysis of the phytochrome A-deficient *fun1* mutants indicates a primary role for phytochrome A in detection of daylength extensions, and hence mediation of the photoperiodic flowering response in pea (Reid et al.1996, Weller et al.1997a, 1997b). Since so many aspects of the AFO5 phenotype are opposite to those of the *fun1* mutants, an analysis of effects on flowering in AFO5 mutants informed by an understanding of the molecular nature of the mutation might be of considerable value. For the time being, this awaits a future breakthrough in identification of the induced molecular lesion present in AFO5 mutant plants.

In the absence of identification of such a molecular lesion, further physiological studies are necessary to gain greater insight into the effects of the AFO5 mutation. A question of some interest is to address the physiological basis of the light-exaggerated phenotype by investigating how phytochrome A is interacting with endogenous plant hormones to regulate stem elongation. Jordan et al.(1995b) describe the correlation of a dwarf phenotype in transgenic tobacco plants overexpressing high levels of oat phytochrome A, with attenuated gibberellin levels (nearly 4-fold reduction). The dwarfing response can be suppressed by foliar applications of GA, and is suggested to result from high levels of PfrA acting to repress GA biosynthesis. This could be examined in AF05, along with phytochrome A/GA interaction in *fun1* mutants, to try to draw the hormone and phytochrome systems together. Pea will be particularly useful for this since it is a species well suited to biochemical studies.

5.4 Materials and methods

5.4.1 Plant material

The dominant AFO5 mutant was selected under FR light following an EMS mutagenesis program conducted in Hobart in 1993. The original mutant was heterozygous but all seed used in the current experiments is from a homozygous mutant line developed by J. Weller.

The phenotype of AFO5 is light-dependent. Occasional slight differences between the phenotype of WT and AFO5 plants grown in the dark-room have been attributed to intermittent exposure to green safelight (Weller 1996). Due to this, care was taken to avoid use of the safelight in experiments involving AF05 wherever possible.

5.4.2 Sequencing of 5' flanking DNA.

Amplification and sequencing of the *PHYA* gene from AFO5 was undertaken in the manner described for the *fun1* mutants (**Chapter 4**). An additional reverse primer (Upstream.seq 5' CTAATTTGGGCAAGTGGGTT 3') was designed to increase coverage of the 5' flanking region. The entire 5545 bp stretch of sequence was sequenced in triplicate from independent DNA preparations in the effort to locate any mutations occurring within the *PHYA* gene of AFO5.

5.4.3 De-etiolation time course experiment

Northern and Western blotting was carried out according to protocols described in **Chapter 3** and **Chapter 4**. The de-etiolation time course experiment was designed so that all plants were of an equivalent age when harvested. Plants were initially grown for seven days in darkness, and then transferred in staggered fashion to a cabinet under R light at 17 $umol^{-2}$ s^{-1}, the same fluence rate used by Weller (1996) when assessing spectrophotometrically detectable phytochrome in AFO5 and WT plants.

Fig. 5.1 Phenotypes of WT (left) and AFO5 (right) mutant plants grown under a range of light environments. All seedlings are 10 days old.

A. Seedlings grown in complete darkness

B. Seedlings grown in continuous W light (150 $\mu mol^{-2}\ s^{-1}$)

C. Seedlings grown in continuous R light (20 $\mu mol^{-2}\ s^{-1}$)

D. Seedlings grown in continuous FR light (8 $\mu mol^{-2}\ s^{-1}$).

The phenotypes are indistinguishable in darkness, but under W, R and FR light the AFO5 seedlings show enhanced repression of stem elongation and increased leaf expansion relative to the WT.

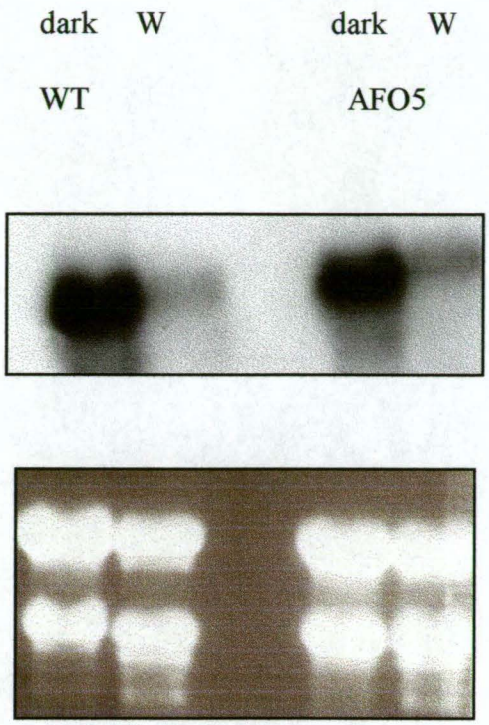

Fig 5.2 *PHYA* mRNA levels in total RNA extracts from WT and AFO5 seedlings, grown for ten days either in either complete darkness or continuous W light. The *PHYA* transcript was detected as a 4.2 kb band. Each lane contains approximately 10 µg total RNA, with the bottom panel of the figure indicating loading of the samples onto the denaturing gel. The probe used to detect the transcript was an 800 bp fragment from the COOH-terminal of a cv. Alaska *PHYA* cDNA clone, kindly provided by Dr. K.I. Tomizawa. Similar results were obtained in three independent experiments.

AFO5 WT AFO5 WT AF05 WT
0 hours 2 hours 4 hours

tubulin loading control:

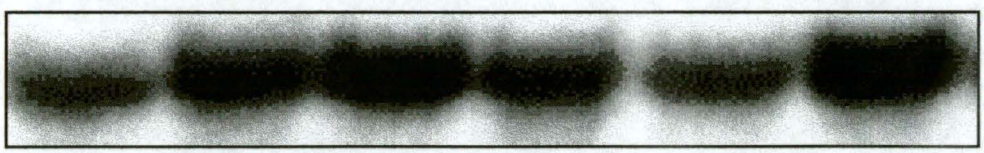

Fig 5.3 *PHYA* mRNA levels in total RNA extracts from WT and AFO5 seedlings. Seedlings were grown for 7 days in darkness and then transferred to R light (17 μmol m^{-2} s^{-1}) for 0, 2 or 4 hours prior to harvest. All seedlings were exactly the same age when harvested. Each lane contains approximately 10 μg of total RNA, and *PHYA* transcript was detected using the probe described in Chapter 4. The major *PHYA* transcript is 4.2 kb (top panel). The bottom panel shows the blot after stripping and re-probing with tubulin as a loading control. Similar results were obtained in two independent experiments.

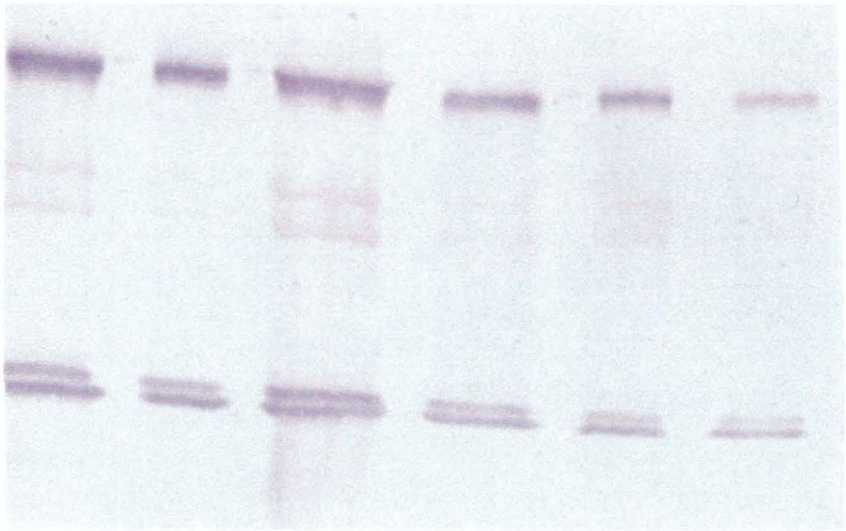

Fig 5.4 PHYA apoprotein levels in crude protein extracts from WT and AFO5 seedlings, grown for 7 days in complete darkness and then transferred to R light (17 μmol $m^{-2}.s^{-1}$) for 0, 2 or 4 hours prior to harvest. Each lane contains protein extract equivalent to 2.5 mg fresh weight. PHYA (top band) is detected as a single band of approximately 121 kD by the monoclonal antibody mAP5 (Nagatani et al. 1984). Similar results were obtained in two independent experiments.

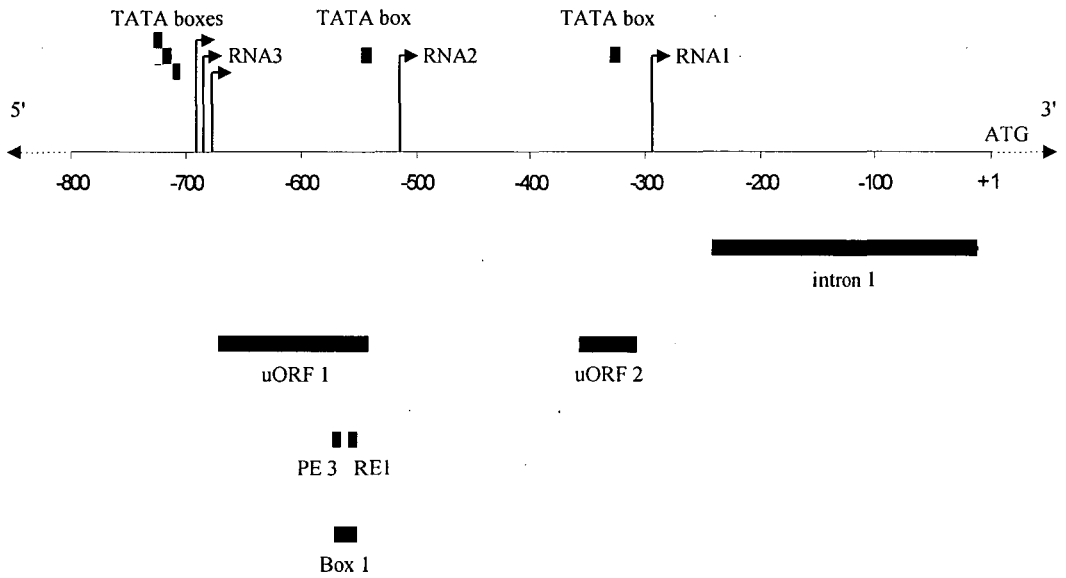

Fig 5.5 A diagram of the 5' flanking region of the pea *PHYA* gene. Locations of the putative TATA boxes and RNA transcription start sites are indicated. The positions of two upstream Open Reading Frames (uORFs) with the potential for translation are shown, plus the first intron. The positions of sequence motifs PE3 (...TCTCCCA...), RE1 (...CATGGG..) and Box 1 (...CCCACACCTTCACATGGG...) are also marked. Adapted from Sato (1988) and Dehesh et al. (1994).

Chapter 6. The effect of FR light on greening ability in pea

6.1 Introduction

On transfer from darkness to W light, many plants undergo a change from the skotomorphogenic to photomorphogenic growth form. This involves alterations in a suite of characters at the morphological, cellular and molecular levels. De-etiolation in a plant such as *Arabidopsis* is characterised by marked inhibition of hypocotyl elongation, unfolding and expansion of the cotyledons, and accumulation of the green photosynthetic pigment chlorophyll. This accumulation of chlorophyll is dependent on the formation of mature chloroplasts in place of the non-green etioplasts of dark-grown seedlings. The developmental programming underlying these changes is complex and likely to be dependent on the interaction of many components. As the photoreceptor responsible for sensing R/FR light, phytochrome plays an essential role in the development of photomorphogenesis.

Germination of *Arabidopsis* seedlings in FR light induces partial photomorphogenic development, but without the accumulation of chlorophyll. Hypocotyl elongation is repressed and the cotyledons open (Whitelam et al. 1993); these are among the classical FR-HIR responses thought to be mediated by phytochrome A (Smith 1995). Barnes et al. (1996) have characterised a FR light response in WT *Arabidopsis* that leads to an inability of the cotyledons to green upon subsequent illumination with W light, due to a phytochrome A-mediated change in plastid development. The FR light block of greening, which can be suppressed by exogenous sucrose, results from severe repression of protochlorophyllide reductase (*POR*) genes by FR light coupled with irreversible plastid damage. POR is required for reduction of the protochlorophyllide pool in non-green etioplasts to chlorophyllide, leading to subsequent chlorophyll production. The block of greening is dependent on the duration of the FR irradiation, is activated during the actual FR treatment, cannot be reversed by subsequent dark treatment, and is insensitive to the W fluence rate on transfer (Barnes et al. 1996a). While phytochrome B-deficient mutants behave in a similar manner to WT plants, mutants deficient in phytochrome A do not lose their ability to green following FR exposure. This indicates a specific role for phytochrome A in mediating the FR block of greening. An intact phytochrome A downstream signalling pathway is also a requirement for the block of greening, as *fhy1* mutants (phytochrome A-specific signal transduction mutants; see Whitelam

et al. 1993, Johnson et al. 1994) green on transfer to FR in a way that WT seedlings are unable to do (Barnes et al. 1996a).

This FR light induced block of greening also occurs in tomato. WT seedlings grown for several days under FR light do not accumulate chlorophyll and fail to undergo cotyledon greening when transferred to W light. Seedling death soon follows (van Tuinen et al. 1995). The phytochrome A-deficient *fri* mutants survive such a transfer, indicating an unambiguous role for phytochrome A in mediation of the block of greening (van Tuinen et al. 1995).

Young pea seedlings have a distinct growth habit from *Arabidopsis* and tomato. *Arabidopsis* and tomato plants are epigeal; the hypocotyl elongates to raise the cotyledons above the ground where they then expand to form the first leaves of the seedling. In contrast, pea is a hypogeal species - the cotyledons remain underground where they act as a nutrient source for the developing epicotyl and radicle. Given these differences, it is relevant to examine the ability of WT pea seedlings to green after exposure to FR light, and to determine whether phytochrome A mutants are affected in this response.

6.2 Results

6.2.1 Qualitative observations of greening ability

The greening ability of WT (cv. Torsdag) pea seedlings was assessed, along with the phytochrome A-deficient mutant *fun1-1* (**Chapter 4**, Weller et al. 1997a), the AFO5 mutant (**Chapter 5**, Weller 1996), the phytochrome B-deficient mutant *lv-5* (Weller et al. 1995) and *fun1-1 lv-5* double mutants.

Seedlings grown under FR light (8 umol $m^{-2}sec^{-1}$) were transferred to daylight conditions in the glasshouse 10 days after planting. After 24 hours, 5 of 7 *fun1-1* seedlings showed some signs of apical greening but 2 seedlings failed to survive, and were characterised by dehydration of the stem below the apex (Fig. 6.1). In contrast with the *fun1-1* plants that struggled to recover from 10 days of etiolated growth under FR, WT and *lv-5* plants in the same experiment developed green leaves and made a successful transition from skotomorphogenic to photomorphogenic growth form.

In a second experiment, the proportion of 12 day-old seedlings which demonstrated successful greening following transfer from FR light

(8 umol $m^{-2}sec^{-1}$) to controlled continuous W light conditions (150 umol $m^{-2}sec^{-1}$) was recorded (Fig. 6.2). As a control, a second batch of seedlings was grown in darkness prior to transfer to W light. Consistent with well-established phenotypes, all pea seedlings grown in the dark were etiolated, with elongated stems, unopened apical hooks and unexpanded leaves. Under FR light WT and *lv-5* seedlings displayed the standard FR-HIR phenotype, with suppression of stem elongation and enhanced leaf expansion relative to the D grown plants. Phytochrome A-deficient *fun1* mutants and *fun1-1 lv-5* double mutants remained fully etiolated under FR, while AFO5 mutants show enhanced de-etiolation compared with the WT.

Following transfer from either D or FR to W, seedlings were visually assessed at 24, 48 and 96 hours post-transfer for signs of healthy greening in the apical region. Etiolated plants moved from the darkroom all appeared healthy 24 hours after transfer, but observation at subsequent timepoints revealed seedling deaths amongst all genotypes (Fig. 6.2). Death of *fun1-1* and *fun1-1 lv-5* seedlings was most prevalent at 48 hours, but by 96 hours after transfer 50% of WT seedlings were dying also.

WT, AFO5 and *lv-5* plants transferred from FR to W light all showed healthy greening over the course of the experiment (Fig. 6.2). In contrast, after 24 hours none of the *fun-1* seedlings preconditioned under FR showed signs of chlorophyll development, although by 48 hours after transfer some greening became apparent. The percentage of *fun1-1 lv-5* plants showing healthy greening was also reduced compared with the WT and other genotypes (Fig. 6.2).

The *fun1-1* and *fun1-1 lv-5* seedlings were etiolated at the time of transfer, regardless of whether they had been preconditioned in darkness or FR light. These genotypes were least able to green, with the most profound effect observed being on *fun1-1* seedlings 24 hours after transfer from FR to W. Although the number of plants included in these experiments was too small for rigorous statistical treatment, some associations between genotype and greening ability were apparent (Fig. 6.2) and further experimentation was warranted.

6.2.2 Seedling death is associated with apparent stem dehydration

It was consistently observed that those plants of various genotypes that failed to survive transfer to W light, either from darkness or FR light, were characterised by dehydration of the stem below the apex (as in Fig. 6.1). The proportion of

plants showing such dehydration, as well as the proportion showing greening of the apex, was recorded for seedlings transferred to W light from FR or darkness 11 days after sowing (Fig. 6.3). Following 4 days exposure to W light, nearly all WT and AFO5 plants accumulated visible amounts of chlorophyll in the apical region (Fig. 6.3A). The majority of *lv-5* seedlings also greened successfully, and the incidence of stem dehydration among these three genotypes was low (Fig. 6.3B). There were no significant differences in greening ability or incidence of stem dehydration for these genotypes ($P>0.05$, Fig. 6.3A,B). In contrast, the percentage of *fun1-1* and *fun1-1 lv-5* plants showing signs of greening of the apex was reduced to 55 – 70 %, while 50 - 60 % showed stem dehydration likely to result in seedling death (Fig. 6.3A, B). Compared with the WT, there was significantly higher failure to green ($P<0.001$, Fig. 6.3A) and significantly increased presence of stem dehydration ($P<0.001$, Fig. 6.3B) among plants of the *fun1-1* and *fun1-1 lv-5* genotypes. This was apparent regardless of initial growing conditions, as there were no significant differences in greening ability or presence of stem dehydration for *fun1-1* or *fun1-1 lv-5* plants preconditioned in D compared with those preconditioned in FR ($P>0.05$, Fig. 6.3A, B).

6.2.3 Chlorophyll accumulation in de-etiolating seedlings

In view of these qualitative observations of greening ability, chlorophyll accumulation following transfer from FR to W light was used as a quantitative measure of greening. Seedlings were transferred to W light 8 days after sowing, and chlorophyll levels were measured at the time of transfer and 36 hours later. Control seedlings grown in darkness before transfer to W light initially showed negligible chlorophyll levels, while those transferred from FR had accumulated trace amounts (Fig. 6.4). After 36 hours in W light, all seedlings transferred from darkness had accumulated similar levels of chlorophyll, with none of the mutants differing significantly from the WT in the amount of chlorophyll detected ($P>0.05$, Fig. 6.4). Among seedlings transferred from FR light, WT, AFO5 and *lv-5* seedlings accumulated chlorophyll equivalent to 284%, 127% and 295% respectively of the levels achieved in the dark preconditioned plants (Fig. 6.4). Partial de-etiolation under FR light presumably makes seedlings of these genotypes more competent for chlorophyll production on transfer to W light than seedlings of the same genotypes transferred from darkness. Although the amount of chlorophyll formed in the apex of WT and *lv-5* plants is approximately the same under these conditions ($P>0.05$, Fig. 6.4), 36 hours after transfer the level of chlorophyll production in AFO5 is significantly lower than in WT seedlings

($P<0.001$, Fig. 6.4). A potentially related observation is that AFO5 plants are much shorter than WT under these conditions ($P<0.001$, data not shown).

FR preconditioned *fun1-1* and *fun1-1 lv-5* seedlings remained etiolated at the time of transfer to W light and after 36 hours had chlorophyll levels equivalent to 96% and 99%, respectively, of those measured in dark-preconditioned plants of the same genotype (Fig. 6.4). These levels are significantly lower than those produced in FR preconditioned WT, AFO5 and *lv-5* seedlings ($P< 0.05$, Fig. 6.4); presumably this is due at least in part to the retention of an etiolated phenotype in *fun1*-type plants grown under FR.

6.3 Discussion

WT pea seedlings transferred to W light from FR generally greened successfully under the experimental conditions. This is distinct from the situation for WT seedlings of *Arabidopsis* and tomato, which fail to green when treated in a similar fashion (van Tuinen et al. 1995, Frick et al. 1995, Barnes et al. 1996a, Runge et al. 1996). Although it has been speculated that the FR-imposed phytochrome-A dependent block of greening described in these two species might be conserved across species (Barnes et al. 1996a, Runge et al. 1996), clearly this does not hold for pea. This may be due to the distinct growth habits of the species; the FR block of greening may be restricted to epigeal species in which the cotyledons are raised above the soil to form the first seedling leaves. It is unclear what role a FR-mediated block of greening might play in an ecological context (Barnes et al. 1996a).

Of the range of pea genotypes considered, *fun1-1* and *fun1-1 lv-5* seedlings are the least capable of greening. This result is the opposite of that seen for tomato and *Arabidopsis* mutants, where phytochrome A deficiency releases seedlings from the FR-imposed block of greening (van Tuinen et al. 1995, Barnes et al. 1996a). The reduced capacity for greening in *fun1-1* and *fun1-1 lv-5* plants is independent of whether seedlings are initially preconditioned with FR light or transferred to W light directly from darkness. Under FR light these genotypes remain etiolated, so the similarity of their greening ability without regard for the preconditioning may be related to this phenotype. However, the etiolated phenotype does not fully explain the decreased greening capacity of these mutants, since etiolated seedlings of other genotypes retain the capacity to develop chlorophyll and in many cases make a successful transition to photomorphogenic growth under the experimental conditions.

Nagatani et al. (1993) found that WT *Arabidopsis* seedlings lose their capacity for greening after prolonged periods of growth in the dark; this may be due to exhaustive utilisation of nutrient resources for hypocotyl elongation. Phytochrome A-deficient mutants of *Arabidopsis* grown initially in darkness also lose greening capacity in a time-dependent manner (Nagatani et al. 1993). These mutants are less able to withstand prolonged dark exposure than the WT, losing greening ability after 3-5 days in darkness, compared with 4-6 days for the WT seedlings. A similar situation may apply in pea. In the experiments described, the mortality of WT control plants (dark grown prior to transfer to W) increased according to the time spent in darkness before transfer. Death rate amongst *fun1-1* plants also increased concomitantly with the length of time elapsed before transfer to W. More detailed examination of the effects of varying lengths of the etiolated growth time may clarify the reduced greening capacity of *fun1* mutants.

Death in the pea seedlings, after transfer from either D or FR, is associated with dehydration of the stem below the apex. A similar phenotype has been observed in WT tomato plants that fail to green on FR to W transfer (H. Kerckhoffs, pers. comm.). A comparative anatomical study of de-etiolating pea seedlings of the various genotypes may provide insight into greening ability. In *Arabidopsis*, ultrastructural analysis over a developmental timecourse indicates that the FR-mediated block of greening is accompanied by failure of plastids to accumulate prolamellar bodies, and ectopic vesicle formation (Barnes et al. 1996).

In conclusion, the FR-induced phytochrome A-dependent repression of greening seen in the epigeal *Arabidopsis* and tomato plants does not occur in pea seedlings. This provides just one example of distinct regulation of development between species of different growth habits, and suggests a need for caution in generalising between species. This highlights the need to examine processes in a diversity of model plant species before pronouncing them as general plant mechanisms. Phytochrome A-deficient pea seedlings do, however, show reduced capacity for greening after a period of etiolated growth in comparison with the WT. This loss of ability to green is not specifically associated with exposure to FR light, as it also occurs in dark grown mutant seedlings. The cause currently remains unknown.

6.4 Methods

6.4.1 Growth conditions

Light sources used were as described in Chapter 3, and all seedlings in these experiments were grown at 20 °C. Greening was quantified by determination of apex total chlorophyll levels using the method of Inskeep and Bloom (1985).

6.4.2 Statistical testing

Differences between means were tested using the students t-test. Testing for association between phenotype and genotype was carried out using 2 x 2 contingency chi-square tests with Yate's correction factor.

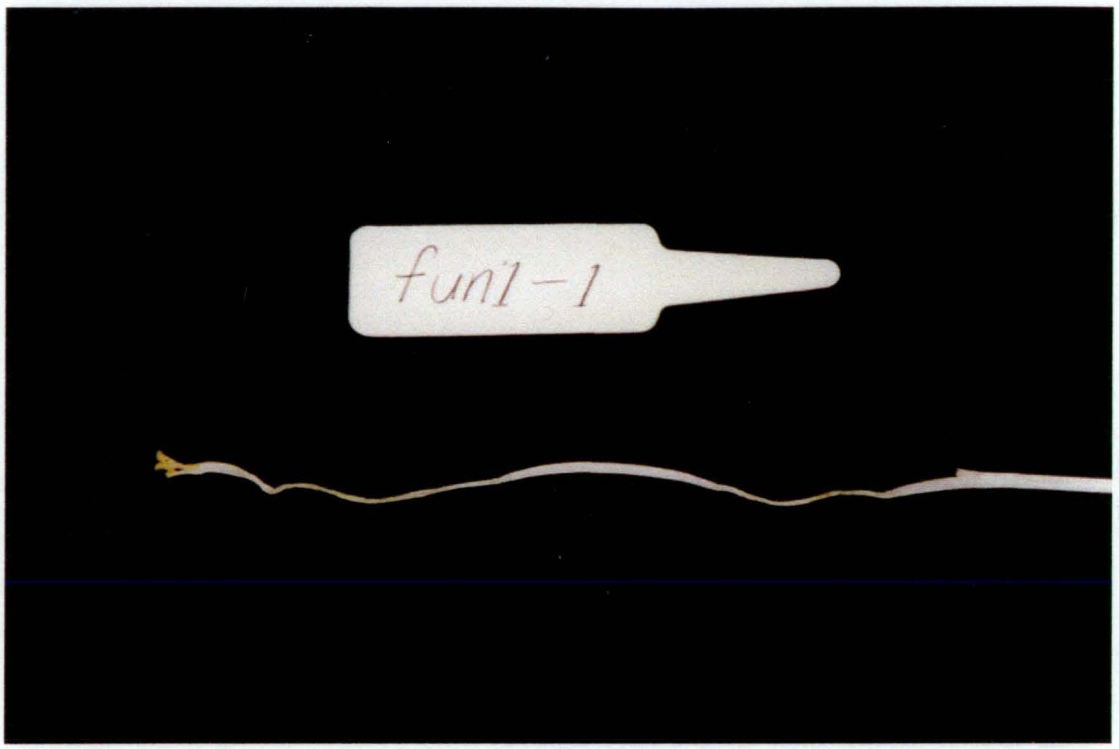

Fig 6.1 Seedling death on transfer was associated with dehydration of the stem below the apex. Shown is a *fun1-1* seedling transferred from FR (8 $umol^{-2}sec^{-1}$) to the glasshouse 10 days after sowing. Within 24 hours of transfer the seedling showed signs of imminent death.

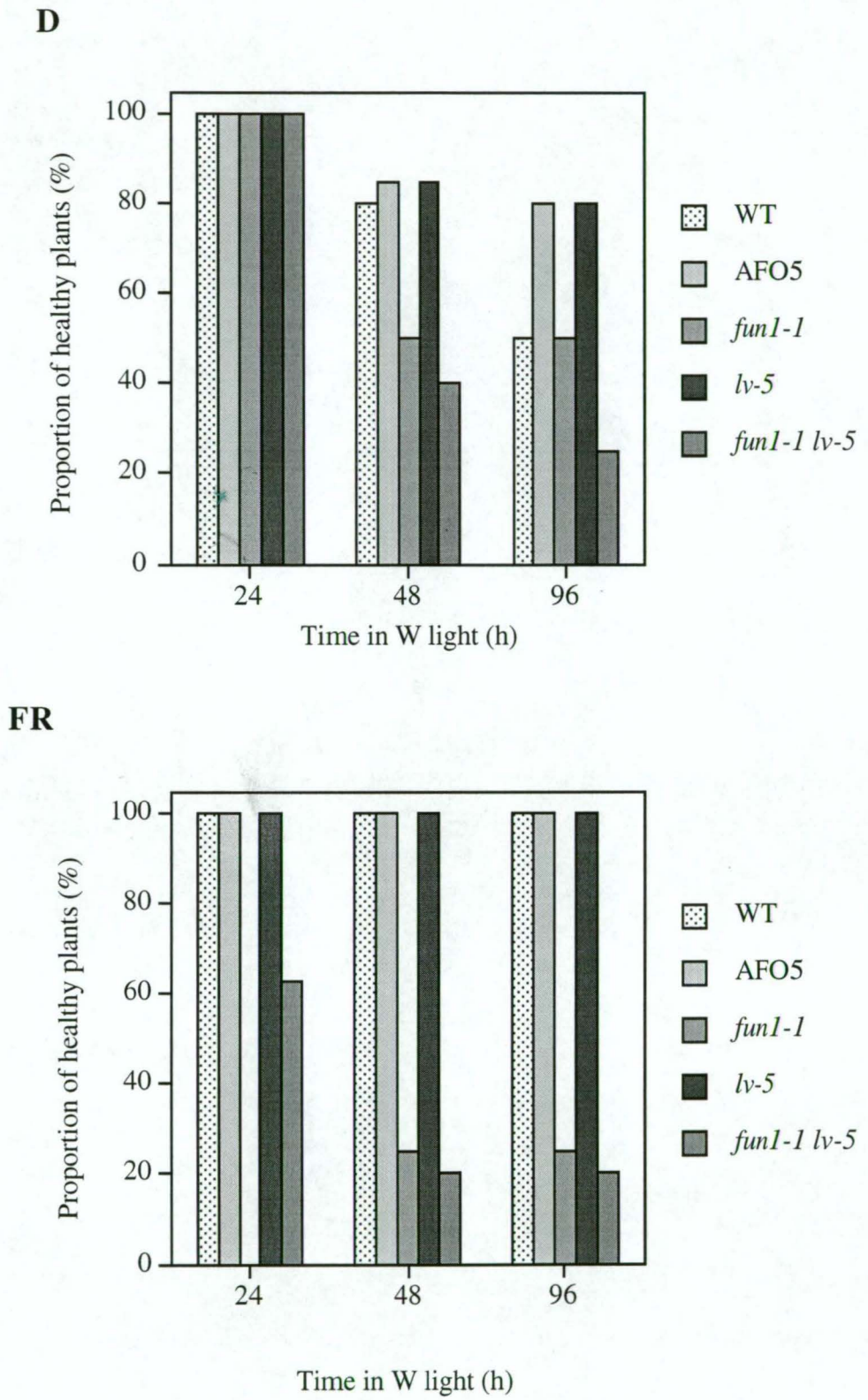

Fig 6.2 Percentage of seedlings showing signs of apical greening 24, 48 and 96 hours after transfer from darkness or FR (8 umol $m^{-2}sec^{-1}$) to W (150 umol $m^{-2}sec^{-1}$) light. All seedlings were 12 days old when transferred. n = 8 – 10. *fun1-1* seedlings preconditioned in FR did not show greening until 48 hours after transfer.

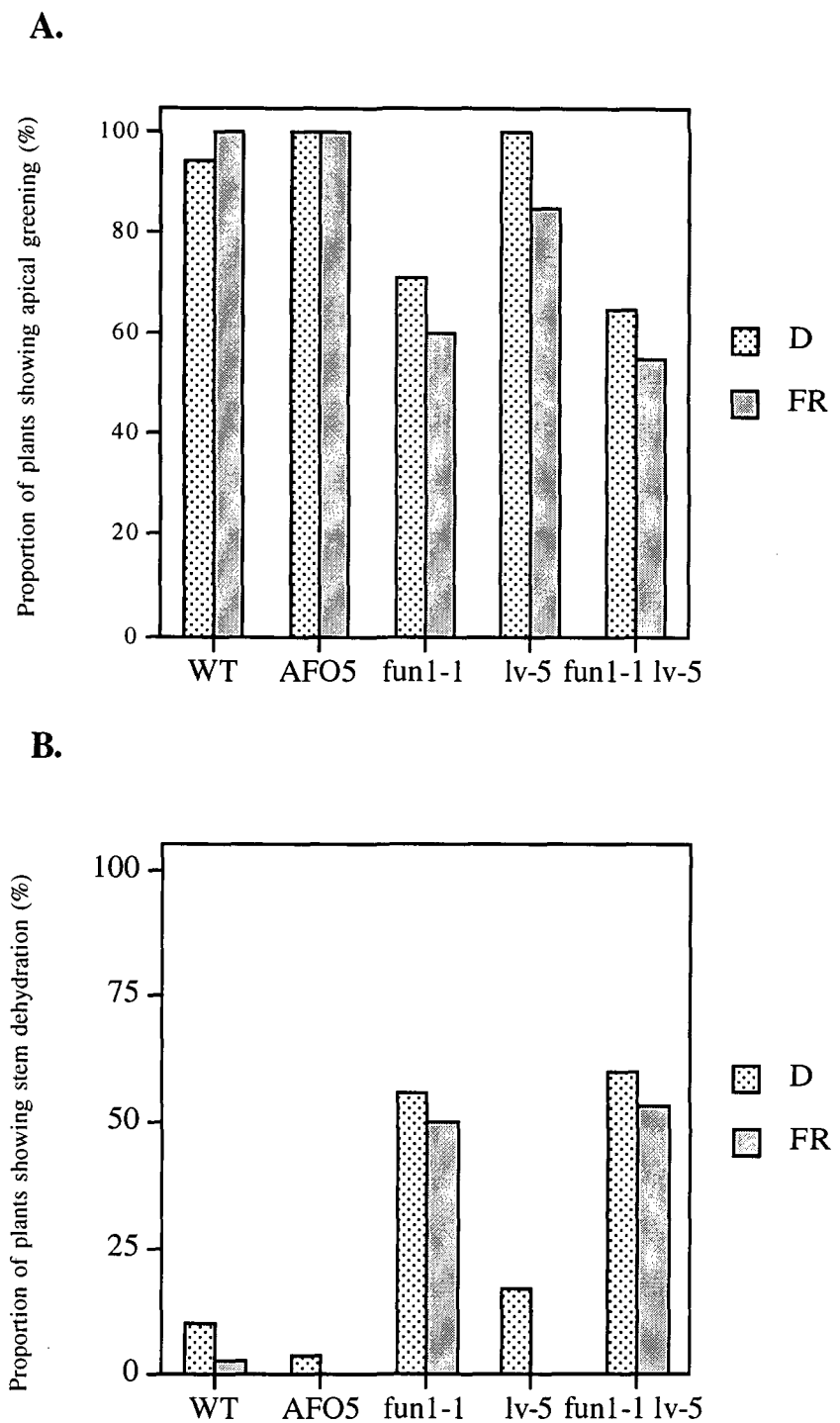

Fig 6.3 Percentage of plants showing (**A**) apical greening and (**B**) stem dehydration, following transfer from darkness or FR (8 umol $m^{-2} sec^{-1}$) to W light (150 umol $m^{-2} sec^{-1}$). All seedlings were 11 days old when transferred. n = 25 – 30.

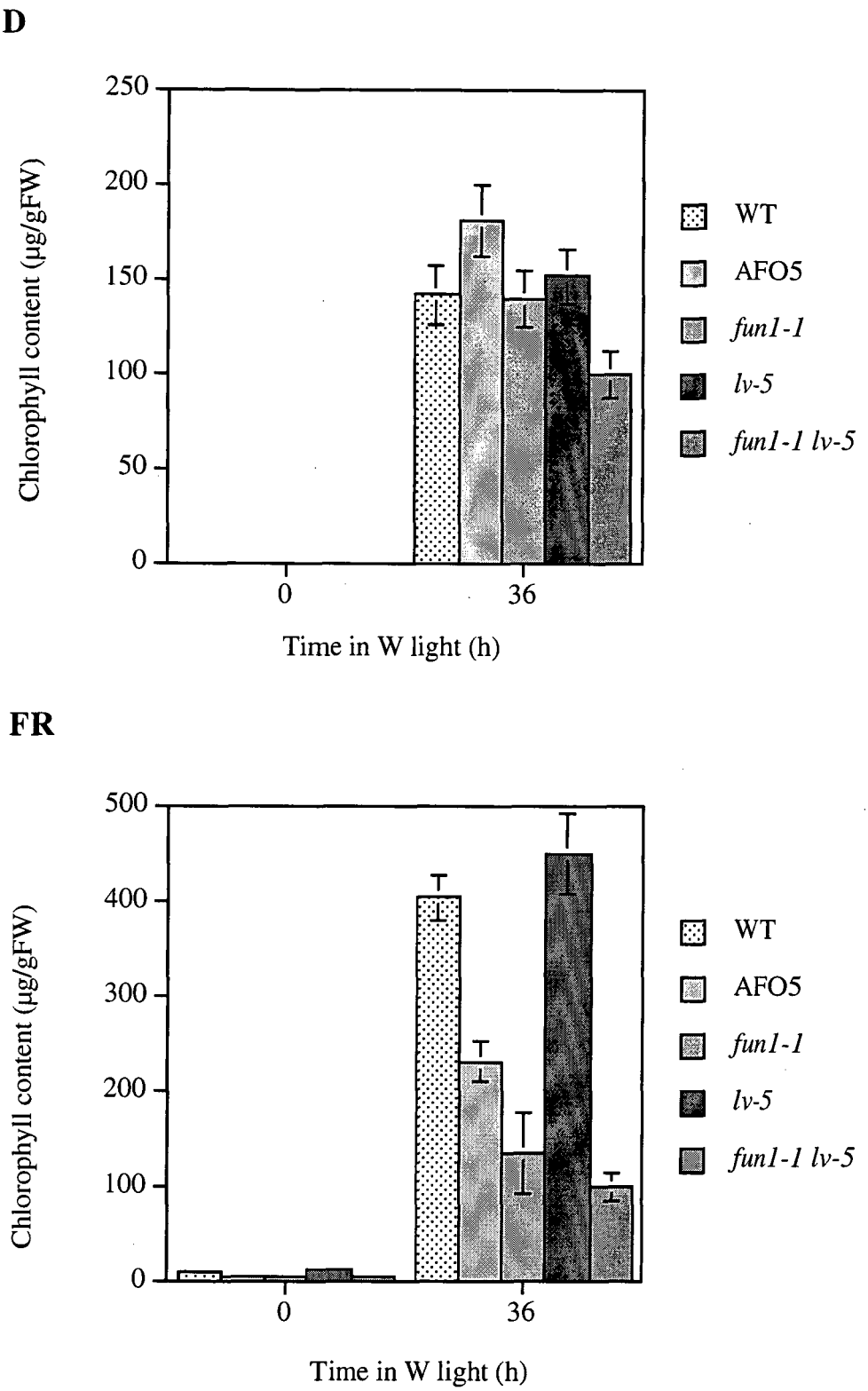

Fig 6.4 Apical chlorophyll content of seedlings transferred to W light (150 umol m^{-2} sec^{-1}) from darkness or FR (8 $umol^{-2}sec^{-1}$). Seedlings were 8 days old at transfer, and chlorophyll measurements were made immediately and 36 hours later. Note the use of different scales, due to the overall level of chlorophyll production in FR-preconditioned plants greatly exceeding that in the dark-preconditioned plants. For chlorophyll determinations, at time zero n = 4 and at 36 hours n = 7 for each genotype. SE are shown.

Chapter 7. General discussion: the phytochrome family in pea

7.1 Phytochrome mutants of pea

Single gene mutants of pea have been identified which are apparently deficient in phytochrome A (*fun1*; Weller et al. 1997a), phytochrome B (*lv*; Weller et al. 1995), and the phytochrome chromophore (*pcd1*, *pcd2*; Weller et al. 1996, 1997c). The AFO5 mutant has characteristics similar to transgenic plants in other species overexpressing phytochrome A (Weller 1996). Further mutagenesis programs are underway to select mutants affected in other phytochromes, and other photoreceptors such as cryptochrome and phototropin (Platten et al. unpub.).

This thesis has established the molecular bases of the *fun1* mutants (**Chapter 4**). The *fun1-1* phenotype results from early introduction of a stop codon after the 530th amino acid of phytochrome A, presumably leading to production of a truncated and inactive holoprotein in the mutant plant. Similarly, the *fun1-2* phenotype is caused by a premature stop codon after the 58th amino acid. Co-segregation of the *fun1-2* mutant phenotype and the molecular lesion identified in the *PHYA* structural gene has been shown. The molecular characterisation of the *fun1* mutants presented in this thesis means they can now be used with confidence in future physiological and biochemical characterisation of the phytochrome family in pea. Parallel studies have identified the molecular bases of phytochrome B-deficiencies in the *lv* mutants of pea (Beauchamp et al. unpub.). Hence, the *fun1* and *lv* mutants can now be used in conjunction to assess the phenotypic effects of simultaneous deficiency of phytochromes A and B. Residual light responses in phytochrome A and B null plants can be attributed to other photoreceptors. It is also possible that mutagenesis on a double mutant background will reveal phenotypes not visible when either phytochrome A or B are present and potentially compensating for, or showing overlapping activity with, other photoreceptors.

This thesis has also investigated the nature of the AFO5 mutation (**Chapter 5**) which leads to a phenotype of exaggerated light responses. Currently the

molecular basis for the phenotype remains elusive as there is no mutation within the *PHYA* structural gene. *PHYA* mRNA levels and PHYA apoprotein levels in AFO5 plants are not markedly different to those seen in WT plants, so further investigation of the molecular cause underlying the phenotype is required.

7.2 The phytochrome family in pea

The precise number of members of the phytochrome family in pea remains to be determined. Currently two phytochromes have been conclusively identified through immunological and molecular studies, phytochrome A (Tomizawa et al. 1986, Sato 1988, Sato 1990) and phytochrome B (Abe et al. 1989, Beauchamp et al. unpub.). Pea mutants doubly deficient in phytochromes A and B (*fun1-1 lv-5*) retain weak de-etiolation under W light (Weller 1996), which indicates possible activity of an additional photoreceptor. Whether this is another member of the phytochrome family, or an as yet unidentified blue light receptor, cannot yet be concluded. Studies from other model species would tend to predict the presence of multiple phytochromes in pea also. In *Arabidopsis* five phytochrome genes are known (Sharrock and Quail 1989, Clack et al. 1995) and as the genome has now been sequenced almost in its entirety, these five are likely to represent the full complement of phytochromes present in the species. Five phytochromes have also been identified in tomato although the *Arabidopsis PHYC* homologue has not been found, and tomato *PHYF* has no direct homologue in *Arabidopsis* (Hauser et al. 1995, 1998).

Low-stringency Southern blotting may give an indication of the number of phytochromes present in pea, although such an approach needs to be used with caution as it may lead to overestimation of family size. For example, Southern data were initially interpreted to indicate the presence of 9 –13 distinct phytochromes in tomato (Hauser et al. 1995). An alternative is to use degenerate oligonucleotide primers to amplify phytochrome fragments for use as probes to screen pea cDNA and genomic libraries for full length clones. While Mathews et al. (1995) have used such primers to amplify phytochrome fragments in a diverse range of species, preliminary attempts to use the same primers for pea were unsuccessful (N. Beauchamp, pers. comm.), and alternative degenerate primer

sets will need to be designed. A third approach would be to use *Arabidopsis PHYC*, *PHYD* and *PHYE* to probe a pea cDNA library for potential homologues. This is how pea *PHYB* was isolated (Beauchamp et al. unpub.).

7.3 Physiological roles of phytochromes in pea

Physiological studies in pea suggest that responses to FR light are mediated by phytochrome A alone, while responses to R light are mediated by both phytochromes A and B (Weller 1996). Responses to blue light, while presumably mediated principally by an as yet unidentified cryptochrome, are influenced by both phytochromes A and B (Weller 1996, Kerckhoffs et al. unpub.). Generally, these results are in keeping with those seen in other model species such as *Arabidopsis* and tomato, but some variations in response have been noted. One example relates to the role of phytochrome A in control of flowering. Phytochrome A-deficient *fun1* mutants grown to maturity under LD show a branched, vegetative phenotype reminiscent of WT plants grown under non-inductive SD. The phytochrome A-deficient plants have reduced capacity to sense FR-rich day extensions, and studies of the *fun1* mutant have established a critical role for phytochrome A in the control of flowering in pea (Weller et al. 1997a). Phytochrome A is considered to mediate flowering by bringing about a reduction in the synthesis or transport of a floral inhibitor (Weller et al. 1997a, 1997b). Similarly, phytochrome A-deficient mutants of *Arabidopsis* show a loss of flowering promotion in response to FR-rich day extension (Johnson et al. 1994). However, extension with white fluorescent light (WFL; containing negligible FR-light) promotes flowering to a normal extent in *Arabidopsis phyA* mutants (Johnson et al. 1994) but for *fun1* plants is even less effective at floral promotion than a FR extension, regardless of whether low or high-fluence is used (Weller et al. 1997a). Future work to further dissect the flowering behaviour of *fun1* mutants is now underpinned by a clear understanding of the molecular cause of the phytochrome A-deficiency in these plants.

Another difference between species is the observation that peas do not show the FR-induced block of greening described for *Arabidopsis* (Barnes et al. 1996) and tomato (van Tuinen et al. 1995)(**Chapter 6**). There are also indications that the

contribution of phytochrome A to blue-light mediated inhibition of stem elongation varies between *Arabidopsis* and pea (Kerckhoffs et al. unpub.). These differences in phytochrome responses and roles between the model species emphasise the need to continue to study a range of species in order to reach valid generalisations about phytochrome activity in plants.

7.4 Future goals in pea phytochrome research

Significant groundwork towards full definition of phytochrome roles in pea has already been done. The range of mutants, and knowledge of the molecular bases of the phytochrome deficiencies they cause, provides an invaluable tool for further work. Molecular characterisation of the *pcd1* and *pcd2* mutants is yet to be carried out, but these genes can be cloned by heterologous library screening using appropriate *Arabidopsis* homologues as they become available. It will also be necessary to fully define the size and identity of members of the phytochrome family present in the genome for pea to reach its full potential as a model.

It is well to remember that phytochromes are responsible for only a portion of plant vision. As mutants in further photoreceptors are uncovered, multiple mutant combinations can be used to assess the interactions between phytochromes and cryptochromes in control of development in pea. Kinetic analysis of the action of photoreceptors in regulating stem growth could also be undertaken, as has been done in other species (e.g. *Arabidopsis*; Parks and Spalding 1999). Further, the well-characterised phytochrome mutants of pea can now be combined with GA mutants to assess the links between phytochrome and hormone pathways in mediating the repression of stem elongation seen when an etiolated pea plant is transferred to a light environment.

The knowledge of pea phytochromes gathered to date, and the potential for future work in this system, highlights the value of using single gene mutants to dissect processes regulating plant growth and development. **Chapter 8** presents another example of the use of such mutants in answering questions about plant development.

Chapter 8. Abscisic acid levels in developing seeds of the gibberellin-deficient mutant *lh-2*

The work presented in this chapter has been published as follows:-
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8.1 Introduction

A seed acts as the dispersal unit for an individual, and correct and complete seed development is of critical importance in transmitting genetic material to the next generation of plants. Development and maturation of the Angiosperm seed is initiated by a double fertilisation event. The Angiosperm seed is comprised of a diploid embryo, produced by fertilisation of an egg cell in the embryo sac by one male pollen tube. There is also a triploid endosperm, formed following fusion of two polar nuclei in the embryo sac with a second pollen tube nucleus. The developing embryo and nutritive endosperm are encased in the seed coat until germination, which begins with imbibition and leads to the elongation of the embryonic axis. Processes involved include protein synthesis and metabolism, subcellular structural changes, respiration, biosynthesis of relevant macromolecules and cell elongation, all in readiness for growth and development of a new individual plant (reviewed in Bewley & Black, 1994).

For these processes to take place in normal and timely fashion, co-ordination of a multi-faceted developmental program is presumably required. Although there is a wealth of information about the latter stages of seed maturation and the germination process (e.g. for reviews see McCarty 1995, Bewley 1997), much less is known about the earliest stages of seed development. One way to advance the understanding of the early events in the formation and control of development in seeds is to identify and characterise mutants with obvious impairments in normal seed development. Well-known examples include the *defective kernel* (*dek*) and *embryo-specific* (*emb*) mutants of maize (Bewley & Black 1994).

In garden pea, mutations at the gibberellin (GA) biosynthesis locus *lh* are associated with an aberrant seed development phenotype, and have previously been used to study the role(s) of GAs in pea seed development. The *lh-2* mutation blocks the three-step oxidation of *ent*-kaurene to *ent*-kaurenoic acid, resulting in a low level of bioactive GA_1 (Swain et al. 1997). Young seeds homozygous for the *lh-2* mutation have reduced levels of some GAs (including GA_1 and GA_3) a few

days after anthesis, when compared with wild-type (WT) seeds, and are more likely to abort during development (Swain et al. 1995). The mutant phenotype is severe, reducing seed weight and decreasing survival by approximately 50% in comparison with the WT (Swain et al. 1997). Many *lh-2* seeds undergo partial development but rather than going on to form germination-capable seeds, remain small and commence desiccation along with neighbouring seeds. Abortion appears to occur at various stages pre-contact point, with no common time point at which development ceases (Swain et al. 1993). Those seeds that do develop to maturity are variable in size and shape, not homogeneous as usually seen for "peas in a pod" (Fig. 8.1). Fertilising mutant seeds with WT pollen restores both GA levels and seed survival (Swain et al. 1995). These results form the basis of the suggestion that GA_1 and/or GA_3 play a significant physiological role in controlling or allowing early development of the embryo/endosperm in garden pea (Swain et al. 1993, 1995, 1997). It appears GAs are not required after contact point, when the liquid endosperm is fully taken up by the embryo (Swain et al. 1997).

It is probable that plant hormones are pivotal in early developmental processes of seeds. The classical plant hormones have well-characterised roles in the control of vegetative growth and development, with examples including the interaction of auxins and cytokinins to regulate canopy structure (branching) and apical dominance (Cline 1994), the stimulation of internode elongation by the gibberellins (GAs) (Ross et al. 1997), and regulation of stomatal closure by abscisic acid (ABA) (Reid and Howell 1995). Similarly, the growth and development of seeds is a complex process, presumably under some form of hormonal regulation. In several studies (e.g. Perata et al. 1990, Piaggesi et al. 1991), seed hormone levels have been shown to fluctuate dramatically according to the stage of development, but the relevance of these changes to seed development is not always clear.

Bewley & Black (1994) suggest that roles for seed hormones may include:
(1) regulation of growth and development, including the arrest of seed growth prior to maturation
(2) regulation of the accumulation and/or utilisation of storage reserves
(3) regulation of growth and development of extra-seminal tissues, i.e. the hormones may be manufactured in the seed for export to other locations in the parent plant
(4) storage for later use in germination and early seedling growth.

The studies of GA levels in the aborting seeds of *lh-2* plants (Swain et al. 1993, 1995, 1997) provide the first strong evidence implicating one of the classical plant hormones in controlling the earliest stages of seed development, with a clear correlation demonstrated between embryo growth and hormone content. There are also several studies indicating roles for, and interactions between, the plant hormones in later stages of seed development and germination. Examples include the interaction of GA and ABA to regulate the activity of hydrolytic enzymes involved in mobilisation of nutrient reserves from the barley aleurone (Jacobsen et al. 1995), and the antagonistic roles of ABA and GA in controlling seed dormancy and germination in some species (reviewed in Bewley 1997). ABA is also implicated in the regulation of accumulation of mRNA transcripts for storage proteins in mid- and late-embryogenesis (reviewed in McCarty 1995).

Although the examples cited provide considerable evidence of a role for ABA in seed maturation, possible roles for this hormone early in development are unclear. ABA has been isolated from immature seeds of many species, including apple (Bewley & Black 1994), bean (Perata et al. 1990), maize (reviewed in Bewley & Black 1994), peach (Piaggesi et al. 1991) and pea (e.g. Wang et al. 1987). It occurs in free form, particularly in legumes, but bound forms such as the glucosyl ester and glucoside are also common. The hormone has been identified in various parts of the seed, including the embryo, the endosperm and seed coats. Major metabolites of ABA – phaseic acid and dihydrophaseic acid – have commonly been identified.

To investigate whether ABA plays a role early in the development of pea seeds, I have examined free ABA levels in young (pre-contact point) seeds, and the interaction between the *lh-2* mutation and *wil*, a mutation which causes a blockage in ABA biosynthesis (Wang et al. 1984). Seeds of *wil* plants generally contain about five times less ABA than WT seeds (de Bruijn et al. 1993) although in pre-contact point seeds the deficiency of ABA can be 10-fold or greater (J. Yaxley, pers. comm.). The *lh-2 wil* double mutant has been constructed in order to examine the effects of this gene combination on pea seed hormone levels and seed development.

8.2 Results

8.2.1 ABA levels in young *lh-2* seeds

Time course experiments were conducted to measure the levels of ABA in young *lh-2* seeds, comprising the testa, liquid endosperm and developing embryo. Two control lines were used, the tall progenitor cv. Torsdag, and the dwarf mutant *ls-1*. The *ls-1* mutant was used as a second control because it is a GA-biosynthesis mutant of similar stature to *lh-2* plants, but the *ls-1* mutation does not appear to reduce GA levels in the embryo or endosperm prior to contact point, and does not affect embryo or endosperm development (Swain et al. 1995). At certain time points after anthesis there was a 3 to 4-fold increase in ABA levels in *lh-2* seeds compared with WT or *ls-1* seeds (Fig. 8.2). This difference has been observed in three separate experiments for the *lh-2* versus WT comparison.

This might be considered a reflection of different growth rates for the seeds of the different genotypes, as ABA levels in older pea seeds show considerable variation during development (Wang et al. 1987). However, when seeds of the same fresh weight – rather than the same age – were compared, increases in ABA levels were also apparent between *lh-2* seeds and WT. The *ls-1* seeds did not exhibit a marked peak in ABA levels like the WT and *lh-2* seeds (Fig. 8.2), and had lower ABA levels than *lh-2* seeds from 5 to 13 days after anthesis.

8.2.2 ABA accumulation is localised in the young *lh-2* seeds

ABA levels in extracts from 10-day-old shoots and roots were measured, to determine whether the accumulation of ABA in young seeds also occurs in vegetative tissues (Table 8.1). Although slight differences in levels were observed between genotypes, there was no evidence of accumulation of ABA as seen in the young seeds. Similar results were obtained in two repeat experiments. There was also no evidence of ABA accumulation in *lh-2* pods. It seems that the increased levels of ABA in pre-contact point *lh-2* seeds are specific to that organ.

8.2.3 Seeds of *lh-2 wil* double mutants have low ABA levels

To investigate whether the accumulation of ABA in *lh-2* seeds is responsible for causing aberrant development in *lh-2* seeds, the *lh-2 wil* double mutant was constructed in order to specifically reduce endogenous ABA levels in *lh-2* seeds. The progeny of a cross between *lh-2* and *wil* plants was grown to identify the *lh-2*

wil double mutant. *lh-2* and *wil* are both single gene recessive mutations (Marx 1976, Swain and Reid 1992). In the F_2 population of 84 plants, 14 putative *wil* plants were identified visually by their wilty phenotype (chi square = 3.1, P>0.05). Identification of selected *wil* segregates was confirmed by growing on from selected F_2 individuals, and harvesting seeds from those F_3 plants for analysis of ABA levels. The levels measured fell into two distinct groups (Fig. 8.3), and confirmed the basis on which genotype had been assigned. Those individuals assigned to the homozygous recessive class contained less than 60 ng ABA g^{-1} fresh weight, while *WIL* – individuals contained more than 220 ng ABA g^{-1} fresh weight.

As the parental *wil* line (L233) is on a *le* (dwarf) background, it was also necessary to distinguish between *le* and *lh-2* dwarf plants in each generation. This was largely possible on the basis of growth form; in particular, pods of *lh-2* plants are shorter and have fewer seeds than those of *le* plants (Swain et al. 1993, 1995). However, to confirm correct identification of *lh-2* types in the F_4, seeds were harvested from selected F_3 plants 24 days after flowering for GA_{20} analysis. At this age seeds of genotype *le* have high levels of GA_{20} while *lh-2* seeds are deficient in this GA (Frydman et al. 1974, Swain et al. 1993). The levels measured fell into two distinct classes on the basis of which genotype was assigned. Extracts from seeds of putative *le* plants contained GA_{20} at levels of 1200 - 2150 ng g^{-1} fresh weight. However, in the *lh-2* samples little or no GA_{20} was detected.

ABA levels were then measured in young seeds of known genotype (Fig. 8.4A). In *lh-2 wil* seeds ABA levels were generally 8 - 10 times lower than in *lh-2 WIL* seeds of the same age. Similar differences were observed between the *le wil* and *le WIL* gene combinations. Therefore, introduction of the *wil* gene reduces endogenous seed ABA levels regardless of the GA status of the seeds or other genes segregating in the cross.

8.2.4 Seed abortion occurs in *lh-2 wil* double mutants

Seed abortion was observed in double mutant plants to the same degree as in *lh-2 WIL* plants (Fig. 8.5). When expressed as a percentage of total seeds produced, nearly 50% of all *lh-2 LE* seeds failed to develop normally (Fig. 8.4B). This was significantly higher than for the *LH le* genotype (P<0.001). However, there was no significant association between abortion and the allele at the *wil* locus (P>0.7).

The 10-fold or greater reduction in ABA levels in pre-contact point *lh-2* seeds brought about by introduction of the *wil* gene (Fig. 8.4A) therefore failed to alleviate the abortion observed in *lh-2 wil* double mutant plants.

8.3 Discussion

The pattern of ABA accumulation in *lh-2* seeds differs from that in the WT progenitor, cv. Torsdag, and in the dwarf mutant *ls-1*. The magnitude and temporal persistence of this difference varies according to whether a comparison is made between hormone levels in seeds of the same chronological age, or seeds at an equivalent physiological stage. The problem in determining which comparison to make arises because seeds of *lh* plants are known to increase in fresh weight more slowly than cv. Torsdag (Swain et al. 1997), and in these experiments *ls-1* seeds also gained in weight slightly more rapidly than *lh-2* seeds. Regardless of which comparison is made, however, the perturbation of ABA levels in *lh-2* seeds is apparent; there seems little doubt that at certain stages, there is more ABA in the *lh-2* seeds. Given the limited current understanding of the role of hormones in the early stages of seed development, it is reasonable to suggest that a transitory hormone imbalance may affect seed development (Zacarías et al. 1995). This line of reasoning is particularly valid given the observation that the accumulation of ABA is specific to the young seeds of *lh-2*, and that one of the most marked aspects of the *lh-2* phenotype is an increase in seed abortion.

Use of the *wil* mutation to manipulate endogenous ABA levels in *lh-2* and *le* plants provides an elegant mechanism for studying the effects of ABA level, and indeed of ABA/GA ratios, in these young seeds, without recourse to applications of synthetic hormones or inhibitors of endogenous synthesis. This is an important aspect of the study, in light of literature detailing unexpected effects of applied substances on hormones other than the one being targeted (e.g. Ross 1998). The introduction of the *wil* gene markedly reduces ABA levels in *lh-2* seeds (Fig. 8.4A), yet fails to restore normal seed development in these plants (Fig. 8.5). This indicates that the variation in ABA levels in early development is not responsible for the marked abortion and reduced seed size that occurs in *lh-2* plants.

These results do not provide an explanation for the increased ABA levels seen at some time points during the early development of *lh-2* seeds. It can be speculated that the blocked conversion of *ent*-kaurene to *ent*-kaurenoic acid in *lh-2* plants (Swain et al. 1997) may alter the flux through pathways beginning with the same

precursors as the GA biosynthesis pathway. In vegetative tissues the GA pathway is generally a minor one and disruption would be unlikely to have large effects on other biosynthesis pathways; this is borne out by the observation that in vegetative tissues of *lh-2* plants GA-deficiency is not accompanied by a concomitant increase in ABA level (Table 8.1). However, the relative importance of the GA pathway appears greater in seeds since GAs reach high levels during late seed development and play a key role in regulating the early development of the embryo/endosperm (Swain et al. 1993, 1995, 1997).

The immediate precursor of *ent*-kaurene, geranylgeranyl diphosphate (GGDP), also serves as a precursor for phytoene, carotenoids and ABA (Fray et al. 1995; Fig. 8.6). Precursors of GGDP are also used in cytokinin synthesis. There are existing accounts of studies where genetic manipulation of one biosynthesis pathway moderates the activity of a second pathway. For example, Fray et al. (1995) reported that overexpression of phytoene synthase (the enzyme regulating the first committed step in carotenoid biosynthesis) in transgenic tomato leads to dwarfism. Measurements revealed that this was due to a 30-fold reduction in GA_1 levels, and was associated with a slight (less than 2-fold) increase in ABA levels and abnormal carotenoid production in a number of organs. Fray et al. (1995) propose that over-production of phytoene synthase leads to excess conversion of GGDP to phytoene, thus diverting this critical intermediate away from the GA biosynthesis pathway. In support of their hypothesis, it has also been shown that antisense inhibition of phytoene synthase expression in tomato causes strong increases in GA levels and slight reduction in ABA levels (Fraser et al. 1995). This explanation of altered flux relies on the assumption that phytoene synthase and kaurene synthase A share a common pool of GGDP (Fraser et al. 1995). An analogous situation may exist in the young GA-deficient *lh-2* seeds, where it may be that an accumulating pool of GGDP, unable to be utilised in GA biosynthesis, is instead shunted into excess ABA production.

Counteracting this suggestion, however, is the report of Domenech et al. (1996) that production of sterols, carotenoids (ABA precursors) and GAs is compartmentalised in the fungus *Gibberella fujikuroi* . In essence, this means that the compounds are synthesised from independent substrate pools. If biosynthesis of these compounds is similarly compartmentalised in plant cells, the concept of a common pool of GGDP cannot be invoked to explain altered flux through the ABA and GA pathways. It has been stated that in plants carotenoids are synthesised in the chloroplasts (Gray 1987), and gibberellins in the cytoplasm using both soluble and membrane-bound enzymes (Hedden 1983, Bearder 1983).

However, Sun and Kamiya (1997) indicate that *ent*-kaurene biosynthesis – the first committed step of the GA biosynthesis pathway – occurs in the plastids, and only the later steps of GA biosynthesis are associated with the endoplasmic reticulum and cytoplasm. If this is the case, the potential for a shared pool of GGDP flowing into various metabolic pathways remains.

The mechanism by which reduced GA levels cause abortion in *lh-2* seeds remains unknown. The earliest indication that a seed will fail to develop is a retarded size related to others in the pod, indicating a possible reduction in assimilate partitioning, or reduced capacity to utilise assimilates (Swain et al. 1997). In particular, embryo development is substantially slowed so that the contact point (the elimination of the liquid endosperm) is much later. Why the different peas in a single pod are so variable in rate and extent of development is also unclear.

One of the reasons for initially suspecting the involvement of a second hormone, such as ABA, in causing the abortion phenotype is that other GA-deficient mutants are not necessarily associated with the same aberrant seed development phenotype. Effects of the less severe allele *lh-1* on seed development are mild and transient (Swain et al. 1997), and the seed phenotype of the GA-deficient *gib1* mutant of tomato is also subtle (Groot et al. 1987). Seed abortion has not been described in any of the *Arabidopsis* GA-deficient mutants to date. One explanation of this apparent paradox revolves around the concept of organ-specific expression. The severe GA-deficiency in *lh-2* seeds may not actually be mirrored in seeds of these other mutants, thus seed development remains relatively unaffected. This is exemplified by the mutant *ls-1*, which has been shown to have reduced shoot GA levels and reduced internode elongation, but normal seed GA levels prior to contact point and normal seed development (Swain et al. 1995).

In conclusion, the effects of *lh-2* on seed development in pea are not mediated through increased ABA levels, as *lh-2 wil* plants exhibit seed abortion yet contain only low levels of ABA in young seeds. It has been demonstrated that introducing the *wil* gene reduces the endogenous ABA level in young (pre-contact point) *lh-2* seeds 10-fold, but this reduction is not associated with a rescue of the seed phenotype. Abortion occurs in the *lh-2 wil* double mutants at the same rate as in the single *lh-2* mutant plants. This indicates that ABA is not responsible for controlling the development of young pea seeds, and is consistent with the theory that GAs regulate development of the embryo/endosperm (Swain et al. 1993, 1995, 1997). The reason for the observed variation of ABA levels in young *lh-2* seeds is unknown at this stage. Precisely how GA deficiency causes seed abortion

is also unknown, although it may be a general consequence of slowed growth rate (Swain et al. 1997).

8.4 Materials and methods

8.4.1 Plant material used in the seed development study

The pure lines of *Pisum sativum* L. used in the experiments are held in the collection at Hobart, Australia. Dwarf lines NGB5843 (*lh-2*; previously lh^i) and L181 (*ls-1*) were derived from the WT tall cv. Torsdag (L107, Reid 1986, Swain and Reid 1992). The *lh-2* mutant has a more severe seed phenotype than the other mutation at this locus (*lh-1*), while of the three *ls* mutations described, *ls-1* is closest to the *lh-2* mutant in stature. Line 233 (*wil*) is on a *r* (wrinkled), *le* (dwarf) background. The original *wil* line was a gift from the late Prof. G. Marx, Cornell University, NY and *wil* is the only mutation described at this locus.

8.4.2 Cultivation of plants

Plants were grown following procedures outlined in **Chapter 3**. Flowers were tagged daily, and only one flower was allowed to develop at each reproductive node. When harvesting young seeds their age was described on the basis of days after flowering, from the day that the flower first opened with the corolla fully reflexed.

Seeds at any one harvest, as far as possible, had developed from flowers which opened and were tagged on the same day. This precaution was included to minimise any effects fluctuations in glasshouse temperatures might have had on flower and seed development. Seeds that were directly compared for hormone content had developed over exactly the same time period under identical temperature conditions.

8.4.3 ABA extraction and analysis

Seeds were harvested between 4 and 14 days after flowering. In all cases, whole seeds and pods were separated from each other immediately after removal from the plant. Harvested tissue was weighed, then immersed in cold methanol containing butylated hydroxytoluene (BHT) and stored at -20°C. For extraction, the concentration of methanol was reduced to 80% by the addition of distilled water. Tissue was then homogenised and filtered and a $^2H_3$-ABA internal

standard (provided by Dr S. Neil, University of Bristol, Bristol, UK) was added to the filtrate.

The filtrate was dried and passed through a preconditioned Sep-Pak C18 cartridge in three 1 ml washes of 0.4% (v/v) acetic acid. The Sep-Pak was washed with a further 2 ml of 0.4% acetic acid, and with 10 ml of 20% methanol in 0.4% acetic acid in water, and the ABA was eluted with 10 ml 35% methanol in 0.4% acetic acid in water. The eluate was reduced to dryness under vacuum, dissolved in 200 µl methanol and methylated with 750 µl ethereal diazomethane, and dried under a stream of nitrogen gas.

The same method was used in extracting ABA from shoot and root tissues harvested from 10-day-old seedlings, except that it was sometimes necessary to add an additional ether partitioning step to further purify the samples. In such cases, the sample was redissolved in 500 µl of distilled water, and partitioned three times against 200 µl of diethyl ether. The ether fraction was dried prior to GC-MS analysis as methyl esters.

Quantification of endogenous ABA was performed using gas chromatography - selected ion monitoring (GC-SIM), using a Kratos Concept ISQ instrument, as described in Hasan et al. (1994). The instrument was operated in the high resolution (R=10 000) mode. The ions monitored for quantification of endogenous ABA were m/z 193.0817 and 190.0629. Identification was confirmed by monitoring additional ions 165.0868 and 162.0680, and by retention time. Endogenous levels were calculated on the basis of peak area, following correction for the presence of unlabelled ABA in the internal standard.

8.4.4 GA extraction and analysis

$^2H_2$-GA_{20} (provided by Prof. L. Mander, Australian National University, Canberra, Australia) was used as an internal standard to monitor endogenous GA levels in seeds harvested 20 days after flowering. Extracts containing GA_{20} were purified using the rapid Sep-Pak method described above for ABA, except that the Sep-Pak was washed with 25% methanol before elution of GA_{20} with 35% methanol.

Quantification of endogenous GA_{20} was performed using GC-MS-SIM, with samples run as methyl ester trimethylsilyl ethers. The ions monitored were m/z 418.2176 and 420.2301. Identification was confirmed by monitoring the

additional ions 375.1628 and 377.1754. Endogenous levels were calculated as before (Ross et al. 1995) on the basis of peak area, following correction for the presence of unlabelled GA in the internal standard and naturally occurring heavy isotopes in the endogenous GA_{20}.

Table 8.1. Harvest details for, and endogenous ABA levels in, extracts from shoot and root tissues from wild-type (WT, cv. Torsdag) and *lh-2* plants. Plants were harvested at 10 days after sowing. The entire aerial portion of 8 plants was used to obtain shoot extracts. Plants were grown in a growth cabinet using a 25 °C 18 h light/ 20 °C 6 h dark cycle. Values shown are means ± SE . For growth measurements n = 15 and for ABA levels n = 2.

Genotype	Leaves expanded	Shoot height (cm)	Tap root (cm)	ABA levels (ng g^{-1} FW) Shoot	Root
WT	5.6 ± 0.1	17.4 ± 0.4	23.0 ± 0.9	5.3 ± 0.1	2.2 ± 0.7
lh-2	4.5 ± 0.1	8.5 ± 0.2	17.4 ± 0.8	3.3 ± 1.2	2.0 ± 0.5

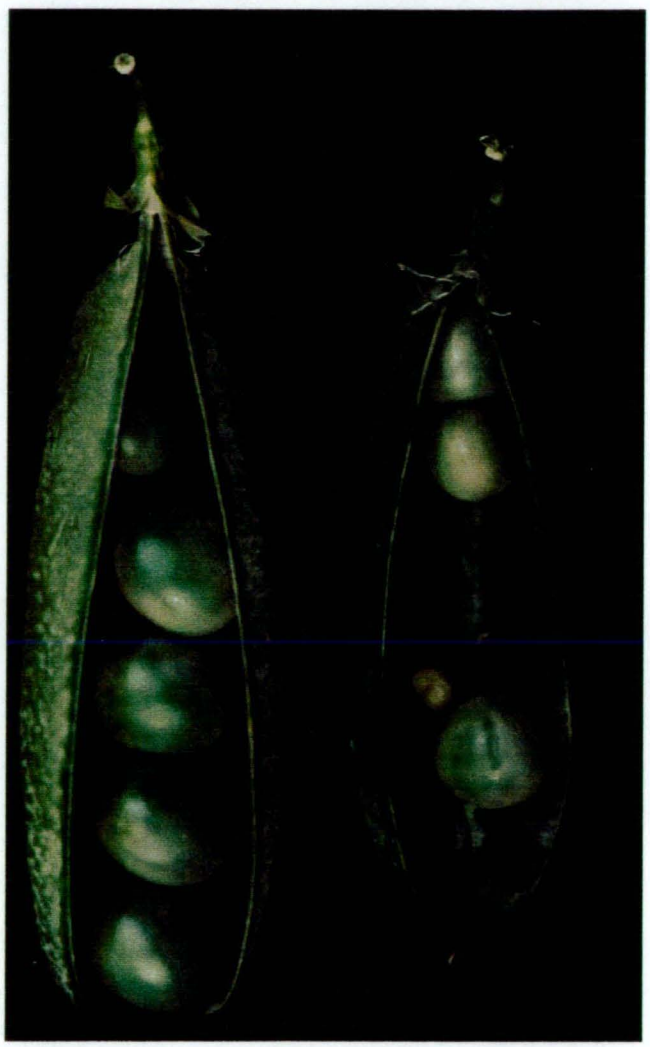

Fig. 8.1 Developing seeds of WT (left) and *lh-2* (right) plants. Note the aborted development and variation in seed size apparent in the pod taken from the *lh-2* plant.

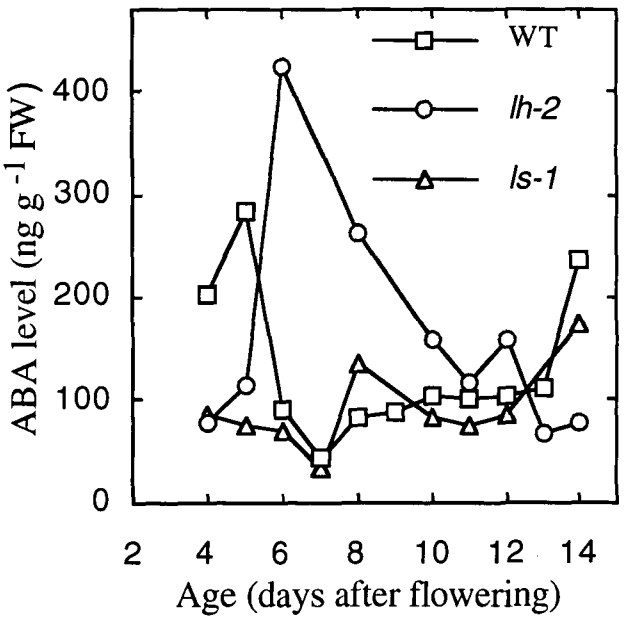

Fig. 8.2 ABA levels in young seeds of genotypes *lh-2*, *ls-1* and WT (cv. Torsdag) from a representative time course experiment. Similar results were obtained in three separate experiments.

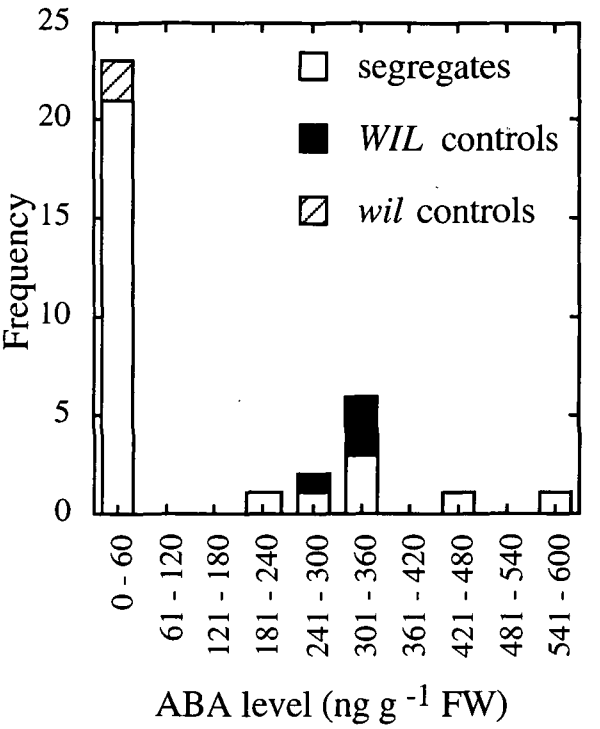

Fig. 8.3 Identification of *wil* and *WIL* - individuals was on the basis of measurement of ABA levels in seeds harvested 20 days after flowering. Those individuals assigned to the homozygous recessive class contained less than 60 ng ABA g^{-1} fresh weight, while *WIL* - individuals contained more than 220 ng ABA g^{-1} fresh weight. ABA levels in parental controls are shown, but these plants were not added to segregates in the analysis.

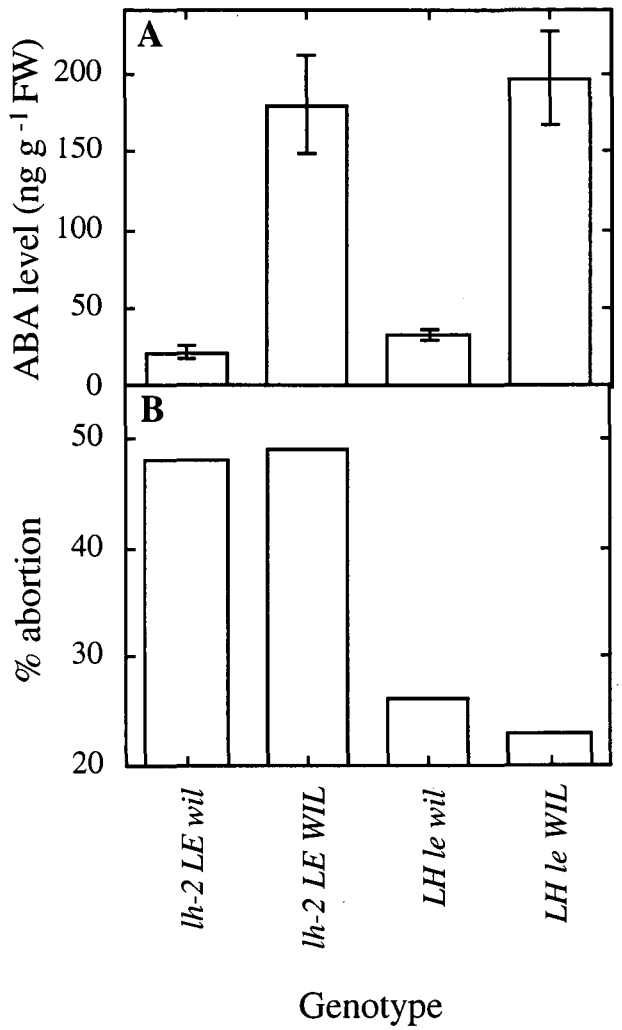

Fig. 8.4 A. ABA levels in young seeds of genotypes *lh-2 LE wil* (n = 5), *lh-2 LE WIL* (n = 5), *LH le wil* (n = 3) and *LH le WIL* (n = 3). Seeds were harvested 6-10 days after flowering. Bars ± SE.

B. Seed abortion, expressed as percentage of total seeds which fail to develop normally, for genotypes *lh-2 LE wil*, *lh-2 LE WIL*, *LH le wil* and *LH le WIL* . The minimum number of seeds assessed for any genotype was 107.

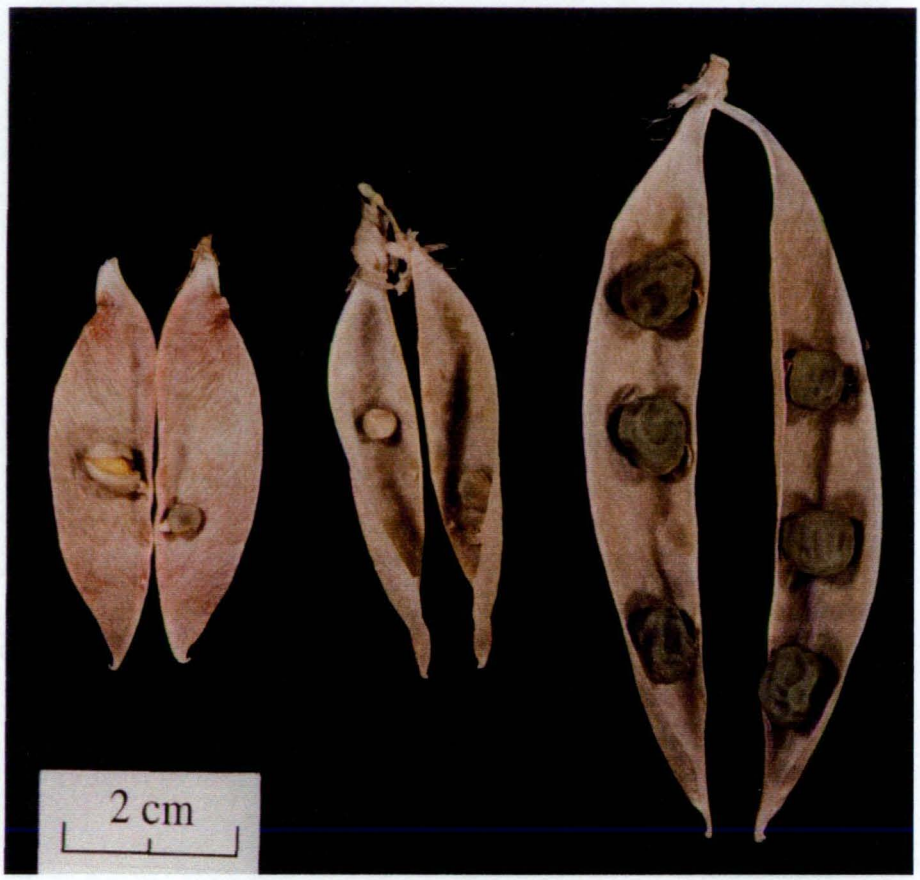

Fig. 8.5 Pods and seeds from the first reproductive node of *lh-2 wil* (left), *lh-2 WIL* (centre) and *le WIL* (right) plants. Seed abortion in the *lh-2 wil* pod is as severe as that seen in the *lh-2 WIL* pod.

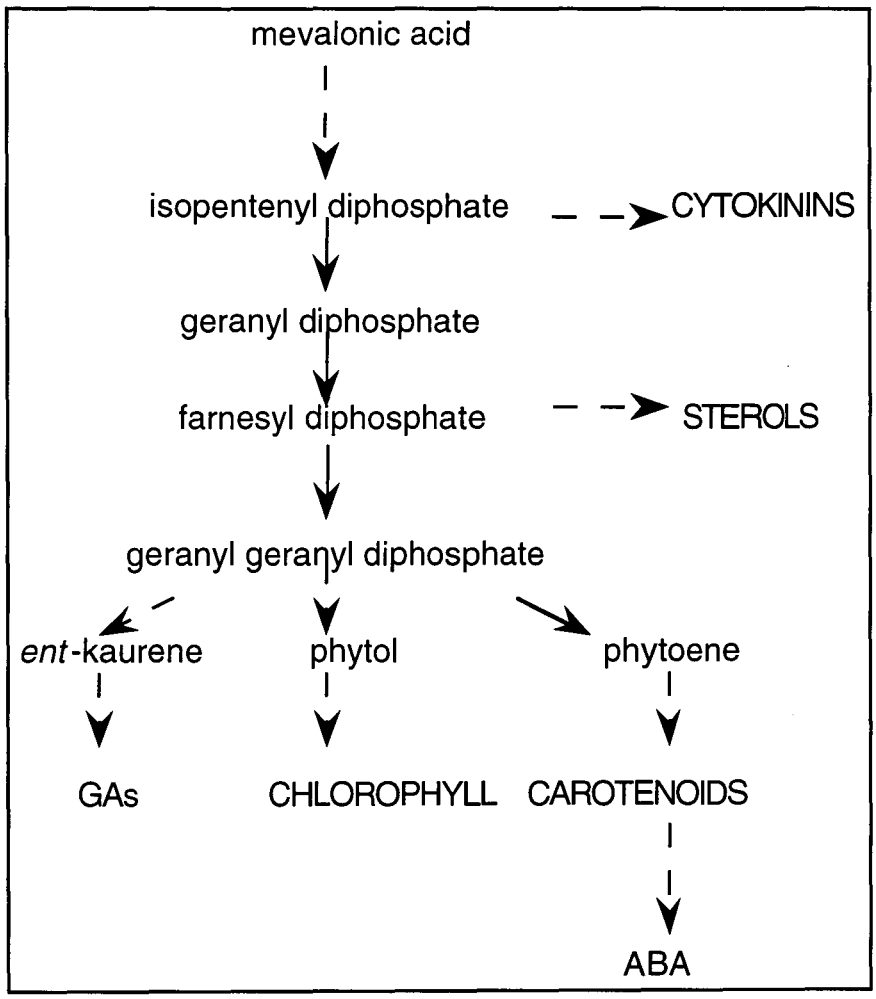

Fig. 8.6 Inter-relationships between the biosynthesis of GAs, carotenoids, ABA and other compounds from a common precursor, geranylgeranyl diphosphate (GGDP). Adapted from Domenech et al. (1996), Fray et al.(1995) and Goodwin and Mercer (1990). A dashed arrow is indicative of a multi-step process.

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